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**WO 03/088970 A2**

(54) Title: MODULATORS OF HEDGEHOG SIGNALING PATHWAYS, COMPOSITIONS AND USES RELATED THERETO

(57) Abstract: The present invention makes available methods and reagents for inhibiting aberrant growth states resulting from hedgehog gain-of-function, ptc loss-of-function or smoothened gain-of-function comprising contacting the cell with a hedgehog antagonist, such as a small molecule, in a sufficient amount to aberrant growth state, e.g., to agonize a normal ptc pathway or antagonize smoothened or hedgehog activity. Such methods and reagents may also inhibit the hedgehog pathway in normal cells, e.g., where normal levels of hedgehog signalling are unwanted.

## Modulators of Hedgehog Signaling Pathways, Compositions and Uses Related Thereto

### Background of the Invention

5        Pattern formation is the activity by which embryonic cells form ordered spatial arrangements of differentiated tissues. The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signaling. Inductive interactions are essential to embryonic patterning in vertebrate development from the earliest establishment of the body plan, to the patterning of the organ systems, to the generation of diverse cell types during tissue differentiation (Davidson, E., (1990) Development 108: 365-389; Gurdon, J. B., (1992) Cell 68: 185-199; Jessell, T. M. et al., (1992) Cell 68: 257-270). The effects of developmental cell interactions are varied. Typically, responding cells are diverted from one route of cell differentiation to another by 10 inducing cells that differ from both the uninduced and induced states of the responding cells (inductions). Sometimes cells induce their neighbors to differentiate like themselves (homeogenetic induction); in other cases a cell inhibits its neighbors from differentiating like itself. Cell interactions in early development may be sequential, such that an initial induction between two cell types leads to a 15 progressive amplification of diversity. Moreover, inductive interactions occur not only in embryos, but in adult cells as well, and can act to establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon (1992) Cell 68:185-199).

20        Members of the *Hedgehog* family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate development. In the fly, a single *hedgehog* gene regulates segmental and 25 imaginal disc patterning. In contrast, in vertebrates, a *hedgehog* gene family is involved in the control of left-right asymmetry, polarity in the CNS, somites and limb, organogenesis, chondrogenesis and spermatogenesis.

The first *hedgehog* gene was identified by a genetic screen in the fruitfly *Drosophila melanogaster* (Nüsslein-Volhard, C. and Wieschaus, E. (1980) *Nature* 287, 795-801). This screen identified a number of mutations affecting embryonic and larval development. In 1992 and 1993, the molecular nature of the *Drosophila* 5 *hedgehog* (*hh*) gene was reported (C.F., Lee et al. (1992) *Cell* 71, 33-50), and since then, several *hedgehog* homologues have been isolated from various vertebrate species. While only one *hedgehog* gene has been found in *Drosophila* and other invertebrates, multiple *Hedgehog* genes are present in vertebrates.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., 10 paralogs of the single drosophila *hedgehog* gene. Exemplary hedgehog genes and proteins are described in PCT publications WO 95/18856 and WO 96/17924. Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, 15 birds, and mammals. A fourth member, herein referred to as tiggle-winkle *hedgehog* (*Thh*), appears specific to fish. Desert hedgehog (*Dhh*) is expressed principally in the testes, both in mouse embryonic development and in the adult rodent and human; Indian hedgehog (*Ihh*) is involved in bone development during embryogenesis and in 20 bone formation in the adult; and, *Shh*, which as described above, is primarily involved in morphogenic and neuroinductive activities. Given the critical inductive roles of hedgehog polypeptides in the development and maintenance of vertebrate organs, the identification of hedgehog interacting proteins is of paramount significance in both clinical and research contexts.

The various *Hedgehog* proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition 25 to signal sequence cleavage in the secretory pathway (Lee, J.J. et al. (1992) *Cell* 71:33-50; Tabata, T. et al. (1992) *Genes Dev.* 2635-2645; Chang, D.E. et al. (1994) *Development* 120:3339-3353), Hedgehog precursor proteins undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee et al. (1994) *Science* 266:1528-1537; Porter et al. (1995) *Nature* 30 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee et al. (1992) *supra*; Tabata et al. (1992) *supra*;

Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Porter *et al.* (1995) *Nature* 374:363; Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Interestingly, cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of HH encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the HH precursor protein proceeds through an internal thioester intermediate that subsequently is cleaved in a nucleophilic substitution. As a result, the C-terminal end 10 of the N-peptide is modified by a cholesterol moiety, tethering it to the cell surface. The biological implications are profound. As a result of the tethering, a high local concentration of N-terminal Hedgehog peptide is generated on the surface of the Hedgehog producing cells. It is this N-terminal peptide which is both necessary and sufficient for short- and long-range Hedgehog signaling activities in *Drosophila* and 15 vertebrates (Porter *et al.* (1995) *supra*; Ekker *et al.* (1995) *supra*; Lai *et al.* (1995) *supra*; Roelink, H. *et al.* (1995) *Cell* 81:445-455; Porter *et al.* (1996) *supra*; Fietz, M.J. *et al.* (1995) *Curr. Biol.* 5:643-651; Fan, C.-M. *et al.* (1995) *Cell* 81:457-465; Mart', E., *et al.* (1995) *Nature* 375:322-325; Lopez-Martinez *et al.* (1995) *Curr. Biol* 5:791-795; Ekker, S.C. *et al.* (1995) *Development* 121:2337-2347; Forbes, A.J. *et* 20 *al.* (1996) *Development* 122:1125-1135).

HH has been implicated in short- and long-range patterning processes at various sites during *Drosophila* development. In the establishment of segment polarity in early embryos, it has short-range effects which appear to be directly mediated, while in the patterning of the imaginal discs, it induces long range effects 25 both directly and via the induction of secondary signals.

In vertebrates, several *hedgehog* genes have been cloned in the past few years. Of these genes, *Shh* has received most of the experimental attention, as it is expressed in different organizing centers that are the sources of signals that pattern neighboring tissues. Recent evidence indicates that *Shh* is involved in these 5 interactions.

The expression of *Shh* starts shortly after the onset of gastrulation in the presumptive midline mesoderm, the node in the mouse (Chang *et al.* (1994) *supra*; Echelard, Y. *et al.* (1993) *Cell* 75:1417-1430), the rat (Roelink, H. *et al.* (1994) *Cell* 76:761-775) and the chick (Riddle, R.D. *et al.* (1993) *Cell* 75:1401-1416), and the 10 shield in the zebrafish (Ekker *et al.* (1995) *supra*; Krauss, S. *et al.* (1993) *Cell* 75:1431-1444). In chick embryos, the *Shh* expression pattern in the node develops a left-right asymmetry, which appears to be responsible for the left-right situs of the heart (Levin, M. *et al.* (1995) *Cell* 82:803-814).

In the CNS, *Shh* from the notochord and the floorplate appears to induce 15 ventral cell fates. When ectopically expressed, *Shh* leads to a ventralization of large regions of the mid- and hindbrain in mouse (Echelard *et al.* (1993) *supra*; Goodrich, L.V. *et al.* (1996) *Genes Dev.* 10:301-312), *Xenopus* (Roelink, H. *et al.* (1994) *supra*; Ruiz i Altaba, A. *et al.* (1995) *Mol. Cell. Neurosci.* 6:106-121), and zebrafish (Ekker *et al.* (1995) *supra*; Krauss *et al.* (1993) *supra*; Hammerschmidt, M., *et al.* 20 (1996) *Genes Dev.* 10:647-658). In explants of intermediate neuroectoderm at spinal cord levels, *Shh* protein induces floorplate and motor neuron development with distinct concentration thresholds, floor plate at high and motor neurons at lower concentrations (Roelink *et al.* (1995) *supra*; Mart' *et al.* (1995) *supra*; Tanabe, Y. *et al.* (1995) *Curr. Biol.* 5:651-658). Moreover, antibody blocking suggests that *Shh* 25 produced by the notochord is required for notochord-mediated induction of motor neuron fates (Mart' *et al.* (1995) *supra*). Thus, high concentration of *Shh* on the surface of *Shh*-producing midline cells appears to account for the contact-mediated induction of floorplate observed *in vitro* (Placzek, M. *et al.* (1993) *Development* 117:205-218), and the midline positioning of the floorplate immediately above the 30 notochord *in vivo*. Lower concentrations of *Shh* released from the notochord and the floorplate presumably induce motor neurons at more distant ventrolateral regions in

a process that has been shown to be contact-independent *in vitro* (Yamada, T. *et al.* (1993) *Cell* 73:673-686). In explants taken at midbrain and forebrain levels, *Shh* also induces the appropriate ventrolateral neuronal cell types, dopaminergic (Heynes, M. *et al.* (1995) *Neuron* 15:35-44; Wang, M.Z. *et al.* (1995) *Nature Med.* 1:1184-1188) 5 and cholinergic (Ericson, J. *et al.* (1995) *Cell* 81:747-756) precursors, respectively, indicating that *Shh* is a common inducer of ventral specification over the entire length of the CNS. These observations raise a question as to how the differential response to *Shh* is regulated at particular anteroposterior positions.

*Shh* from the midline also patterns the paraxial regions of the vertebrate 10 embryo, the somites in the trunk (Fan *et al.* (1995) *supra*) and the head mesenchyme rostral of the somites (Hammerschmidt *et al.* (1996) *supra*). In chick and mouse paraxial mesoderm explants, *Shh* promotes the expression of sclerotome specific markers like *Pax1* and *Twist*, at the expense of the dermamyotomal marker *Pax3*. Moreover, filter barrier experiments suggest that *Shh* mediates the induction of the 15 sclerotome directly rather than by activation of a secondary signaling mechanism (Fan, C.-M. and Tessier-Lavigne, M. (1994) *Cell* 79, 1175-1186).

*Shh* also induces myotomal gene expression (Hammerschmidt *et al.* (1996) *supra*; Johnson, R.L. *et al.* (1994) *Cell* 79:1165-1173; Münsterberg, A.E. *et al.* (1995) *Genes Dev.* 9:2911-2922; Weinberg, E.S. *et al.* (1996) *Development* 20 122:271-280), although recent experiments indicate that members of the WNT family, vertebrate homologues of *Drosophila wingless*, are required in concert (Münsterberg *et al.* (1995) *supra*). Puzzlingly, myotomal induction in chicks requires higher *Shh* concentrations than the induction of sclerotomal markers (Münsterberg *et al.* (1995) *supra*), although the sclerotome originates from somitic cells positioned 25 much closer to the notochord. Similar results were obtained in the zebrafish, where high concentrations of Hedgehog induce myotomal and repress sclerotomal marker gene expression (Hammerschmidt *et al.* (1996) *supra*). In contrast to amniotes, however, these observations are consistent with the architecture of the fish embryo, as here, the myotome is the predominant and more axial component of the somites. 30 Thus, modulation of *Shh* signaling and the acquisition of new signaling factors may have modified the somite structure during vertebrate evolution.

In the vertebrate limb buds, a subset of posterior mesenchymal cells, the "Zone of polarizing activity" (ZPA), regulates anteroposterior digit identity (reviewed in Honig, L.S. (1981) *Nature* 291:72-73). Ectopic expression of *Shh* or application of beads soaked in *Shh* peptide mimics the effect of anterior ZPA grafts, 5 generating a mirror image duplication of digits (Chang *et al.* (1994) *supra*; Lopez-Martinez *et al.* (1995) *supra*; Riddle *et al.* (1993) *supra*) (Fig. 2g). Thus, digit identity appears to depend primarily on *Shh* concentration, although it is possible that other signals may relay this information over the substantial distances that appear to be required for AP patterning (100-150  $\mu$ m). Similar to the interaction of 10 HH and DPP in the *Drosophila* imaginal discs, *Shh* in the vertebrate limb bud activates the expression of *Bmp2* (Francis, P.H. *et al.* (1994) *Development* 120:209-218), a *dpp* homologue. However, unlike DPP in *Drosophila*, *Bmp2* fails to mimic the polarizing effect of *Shh* upon ectopic application in the chick limb bud (Francis *et al.* (1994) *supra*). In addition to anteroposterior patterning, *Shh* also appears to be 15 involved in the regulation of the proximodistal outgrowth of the limbs by inducing the synthesis of the fibroblast growth factor FGF4 in the posterior apical ectodermal ridge (Laufer, E. *et al.* (1994) *Cell* 79:993-1003; Niswander, L. *et al.* (1994) *Nature* 371:609-612).

The close relationship between Hedgehog proteins and BMPs is likely to 20 have been conserved at many, but probably not all sites of vertebrate *Hedgehog* expression. For example, in the chick hindgut, *Shh* has been shown to induce the expression of *Bmp4*, another vertebrate *dpp* homologue (Roberts, D.J. *et al.* (1995) *Development* 121:3163-3174). Furthermore, *Shh* and *Bmp2*, 4, or 6 show a striking correlation in their expression in epithelial and mesenchymal cells of the stomach, 25 the urogenital system, the lung, the tooth buds and the hair follicles (Bitgood, M.J. and McMahon, A.P. (1995) *Dev. Biol.* 172:126-138). Further, *Ihh*, one of the two other mouse *Hedgehog* genes, is expressed adjacent to *Bmp* expressing cells in the gut and developing cartilage (Bitgood and McMahon (1995) *supra*).

Recent evidence suggests a model in which *Ihh* plays a crucial role in the 30 regulation of chondrogenic development (Roberts *et al.* (1995) *supra*). During cartilage formation, chondrocytes proceed from a proliferating state via an

intermediate, prehypertrophic state to differentiated hypertrophic chondrocytes. *Ihh* is expressed in the prehypertrophic chondrocytes and initiates a signaling cascade that leads to the blockage of chondrocyte differentiation. Its direct target is the perichondrium around the *Ihh* expression domain, which responds by the expression 5 of *Gli* and *Patched* (*Ptc*), conserved transcriptional targets of Hedgehog signals (see below). Most likely, this leads to secondary signaling resulting in the synthesis of parathyroid hormone-related protein (PTHrP) in the periarticular perichondrium. PTHrP itself signals back to the prehypertrophic chondrocytes, blocking their further differentiation. At the same time, PTHrP represses expression of *Ihh*, thereby 10 forming a negative feedback loop that modulates the rate of chondrocyte differentiation.

*Patched* was originally identified in *Drosophila* as a segment polarity gene, one of a group of developmental genes that affect cell differentiation within the individual segments that occur in a homologous series along the anterior-posterior 15 axis of the embryo. See Hooper, J.E. et al. (1989) *Cell* 59:751; and Nakano, Y. et al. (1989) *Nature* 341:508. Patterns of expression of the vertebrate homologue of *patched* suggest its involvement in the development of neural tube, skeleton, limbs, craniofacial structure, and skin.

Genetic and functional studies demonstrate that *patched* is part of the 20 hedgehog signaling cascade, an evolutionarily conserved pathway that regulates expression of a number of downstream genes. See Perrimon, N. (1995) *Cell* 80:517; and Perrimon, N. (1996) *Cell* 86:513. *Patched* participates in the constitutive transcriptional repression of the target genes; its effect is opposed by a secreted glycoprotein, encoded by hedgehog, or a vertebrate homologue, which induces 25 transcriptional activation. Genes under control of this pathway include members of the Wnt and TGF-beta families.

*Patched* proteins possess two large extracellular domains, twelve transmembrane segments, and several cytoplasmic segments. See Hooper, *supra*; Nakano, *supra*; Johnson, R.L. et al. (1996) *Science* 272:1668; and Hahn, H. et al. 30 (1996) *Cell* 85:841. The biochemical role of *patched* in the hedgehog signaling

pathway is unclear. Direct interaction with the hedgehog protein has, however, been reported (Chen, Y. et al. (1996) *Cell* 87:553), and *patched* may participate in a hedgehog receptor complex along with another transmembrane protein encoded by the *smoothened* gene. See Perrimon, *supra*; and Chen, *supra*. However, other studies 5 have shown *patched* appears to act catalytically, and only indirectly suppresses *smoothened* activity (see, e.g., Taipale et al. (2002) *Nature* 418, 892-896).

The human homologue of *patched* was recently cloned and mapped to chromosome 9q22.3. See Johnson, *supra*; and Hahn, *supra*. This region has been implicated in basal cell nevus syndrome (BCNS), which is characterized by 10 developmental abnormalities including rib and craniofacial alterations, abnormalities of the hands and feet, and spina bifida.

BCNS also predisposes to multiple tumor types, the most frequent being basal cell carcinomas (BCC) that occur in many locations on the body and appear within the first two decades of life. Most cases of BCC, however, are unrelated to 15 the syndrome and arise sporadically in small numbers on sun-exposed sites of middle-aged or older people of northern European ancestry.

Recent studies in BCNS-related and sporadic BCC suggest that a functional loss of both alleles of *patched* leads to development of BCC. See Johnson, *supra*; Hahn, *supra*; and Gailani, M.R. et al. (1996) *Nature Genetics* 14:78. Single allele 20 deletions of chromosome 9q22.3 occur frequently in both sporadic and hereditary BCC. Linkage analysis revealed that the defective inherited allele was retained and the normal allele was lost in tumors from BCNS patients.

Sporadic tumors also demonstrated a loss of both functional alleles of *patched*. Of twelve tumors in which *patched* mutations were identified with a single 25 strand conformational polymorphism screening assay, nine had chromosomal deletion of the second allele and the other three had inactivating mutations in both alleles (Gailani, *supra*). The alterations did not occur in the corresponding germline DNA.

Most of the identified mutations resulted in premature stop codons or frame 30 shifts. Lench, N.J., et al., *Hum. Genet.* 1997 Oct; 100(5-6): 497-502. Several,

however, were point mutations leading to amino acid substitutions in either extracellular or cytoplasmic domains. These sites of mutation may indicate functional importance for interaction with extracellular proteins or with cytoplasmic members of the downstream signaling pathway.

5 The involvement of *patched* in the inhibition of gene expression and the occurrence of frequent allelic deletions of *patched* in BCC support a tumor suppressor function for this gene. Its role in the regulation of gene families known to be involved in cell signaling and intercellular communication provides a possible mechanism of tumor suppression.

10

### Summary of the Invention

The present invention makes available methods and reagents for inhibiting activation of the hedgehog signaling pathway, e.g., to inhibit aberrant growth states resulting from phenotypes such as *ptc* loss-of-function, *hedgehog* gain-of-function, 15 or *smoothened* gain-of-function, comprising contacting the cell with an agent, such as a small molecule, in a sufficient amount to agonize a *ptc* activity, antagonize a *hedgehog* activity, or antagonize *smoothened* activity, e.g., to reverse or control the aberrant growth state. The present invention contemplates *hedgehog* antagonists that inhibit the *hedgehog* pathway in a *smoothened* gain-of-function cell with an IC<sub>50</sub> 20 within a factor of 20, 10, or even a factor of 5, of the IC<sub>50</sub> with which they inhibit the *hedgehog* pathway in a *patched*-null cell.

### Detailed Description of the Invention

#### *I. Overview*

25 The present invention relates to the discovery that signal transduction pathways regulated by *hedgehog*, *patched* (*ptc*), *gli* and/or *smoothened* can be inhibited, at least in part, by small molecules. While not wishing to be bound by any particular theory, the activation of a receptor may be the mechanism by which these agents act. For example, the ability of these agents to inhibit proliferation of *patched*

loss-of-function (*ptc*<sup>lof</sup>) cells may be due to the ability of such molecules to interact with *hedgehog*, *patched*, or *smoothened*, or at least to interfere with the ability of those proteins to activate a *hedgehog*, *ptc*, and/or *smoothened*-mediated signal transduction pathway. Experiments relating to small molecule inhibitors of the 5 *hedgehog* pathway are discussed in Chen et al., Proc. Nat. Acad. Sci. 2002, 99:22, 14071-14076; Taipale et al., Nature 2002, 418, 892-897; and Taipale et al., Nature 2000, 406, 1005-1009, all of which are incorporated herein by reference in their entirety. These studies suggest that *hedgehog* antagonists of different structures, even ones that bind to the same protein in the *hedgehog* pathway, may act in slightly 10 different ways. Accordingly, even if a particular condition caused or contributed to by aberrant or unwanted activation of the *hedgehog* pathway shows little response to treatment by one of the antagonists disclosed herein, another of the antagonists disclosed herein may nonetheless be efficacious.

It is, therefore, specifically contemplated that these small molecules which 15 interfere with aspects of *hedgehog*, *ptc*, or *smoothened* signal transduction activity will likewise be capable of inhibiting proliferation (or other biological consequences) in normal cells and/or cells having a *patched* loss-of-function phenotype, a *hedgehog* gain-of-function phenotype, or a *smoothened* gain-of-function phenotype. Thus, it is contemplated that in certain embodiments, these 20 compounds may be useful for inhibiting *hedgehog* activity in normal cells, e.g., which do not have a genetic mutation that activates the *hedgehog* pathway. In preferred embodiments, the subject inhibitors are organic molecules having a molecular weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less than 750 amu, and are capable of inhibiting at least some of the 25 biological activities of hedgehog proteins, preferably specifically in target cells.

Thus, the methods of the present invention include the use of small molecules which agonize *ptc* inhibition of *hedgehog* signalling, such as by inhibiting activation of *smoothened* or downstream components of the signal pathway, in the regulation of repair and/or functional performance of a wide range of cells, tissues 30 and organs, including normal cells, tissues, and organs, as well as those having the phenotype of *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-

of-function. For instance, the subject method has therapeutic and cosmetic applications ranging from regulation of neural tissues, bone and cartilage formation and repair, regulation of spermatogenesis, regulation of smooth muscle, regulation of lung, liver and other organs arising from the primitive gut, regulation of 5 hematopoietic function, regulation of skin and hair growth, etc. Moreover, the subject methods can be performed on cells that are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*). See, for example, PCT publications WO 95/18856 and WO 96/17924 (the specifications of which are expressly incorporated by reference herein).

10 In a preferred embodiment, the subject method can be to treat epithelial cells having a phenotype of *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function. For instance, the subject method can be used in treating or preventing basal cell carcinoma or other *hedgehog* pathway-related disorders.

15 In certain embodiments, a subject antagonist may inhibit activation of a *hedgehog* pathway by binding to *smoothened*.

In another preferred embodiment, the subject method can be used as part of a treatment regimen for one or more of: malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors (Berman et al. *Science*, 2002), 20 rhabdomyosarcoma, lung cancer (in particular, small cell lung cancer), gut-derived tumors (including but not limited to cancer of the esophagus, stomach, pancreas, and biliary duct system), and prostatic and bladder cancers.

In another aspect, the present invention provides pharmaceutical preparations comprising, as an active ingredient, a *hedgehog* antagonist, *ptc* agonist, or 25 *smoothened* antagonist such as described herein, formulated in an amount sufficient to inhibit, *in vivo*, proliferation or other biological consequences of *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function.

The subject treatments using *hedgehog* antagonists, *patched* agonists, or *smoothened* antagonists can be effective for both human and animal subjects.

Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs, and goats.

## *II. Definitions*

5 For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

10 The phrase "aberrant modification or mutation" of a gene refers to such genetic lesions as, for example, deletions, substitution or addition of nucleotides to a gene, as well as gross chromosomal rearrangements of the gene and/or abnormal methylation of the gene. Likewise, mis-expression of a gene refers to aberrant levels of transcription of the gene relative to those levels in a normal cell under similar conditions, as well as non-wild-type splicing of mRNA transcribed from the gene.

15 "Basal cell carcinomas" exist in a variety of clinical and histological forms such as nodular-ulcerative, superficial, pigmented, morphealike, fibroepithelioma and nevoid syndrome. Basal cell carcinomas are the most common cutaneous neoplasms found in humans. The majority of new cases of nonmelanoma skin cancers fall into this category.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

20 The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma", which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; 25 "epidermoid carcinoma", which refers to cancerous cells which tend to differentiate 30

in the same way as those of the epidermis; i.e., they tend to form prickle cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of 5 tubular cells in varying arrangements. Other carcinomatous epithelial growths are "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

10 The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

15 "Dental tissue" refers to tissue in the mouth that is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

20 "Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers that can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

25 The term "ED<sub>50</sub>" means the dose of a drug that produces 50% of its maximum response or effect.

30 An "effective amount" of, e.g., a *hedgehog* antagonist, with respect to the subject method of treatment, refers to an amount of the antagonist in a preparation which, when applied as part of a desired dosage regimen brings about, e.g., a change in the rate of cell proliferation and/or the state of differentiation of a cell and/or rate

of survival of a cell according to clinically acceptable standards for the disorder to be treated or the cosmetic purpose.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous),  
5 including the glands and other structures derived therefrom, e.g., corneal, esophageal, epidermal, and hair follicle epithelial cells. Other exemplary epithelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells;  
10 squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, like that which is characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g., tissue which represents a transition between stratified squamous and columnar epithelium.

15 The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in  
20 the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm.  
25 On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and

horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

“Excisional wounds” include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into 5 subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

The “growth state” of a cell refers to the rate of proliferation of the cell and/or the state of differentiation of the cell. An “altered growth state” is a growth state characterized by an abnormal rate of proliferation, e.g., a cell exhibiting an 10 increased or decreased rate of proliferation relative to a normal cell.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, “hair” may refer to the aggregate of such hairs. A "hair follicle" refers to one 15 of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow. "Hair follicle epithelial cells" refers to epithelial cells that surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

20 The term “*hedgehog* antagonist” refers to an agent that inhibits *hedgehog* signalling, such as to repress transcription of target genes or regulate interactions among proteins and/or signalling molecules. Preferred *hedgehog* antagonists can be used to overcome a *ptc* loss-of-function and/or a *smoothened* gain-of-function, the latter also being referred to as *smoothened* antagonists. The term ‘*hedgehog* 25 antagonist’ as used herein refers not only to any agent that may act by directly inhibiting the normal function of the *hedgehog* protein, but also to any agent that inhibits the *hedgehog* signalling pathway at any level, e.g., recapitulates the function of *ptc* or blocks the signalling pathway at a downstream location.

30 The term “*hedgehog* gain-of-function” refers to an aberrant modification or mutation of a *ptc* gene, *hedgehog* gene, or *smoothened* gene, or a decrease (or loss)

in the level of expression of such a gene, which results in a phenotype which resembles contacting a cell with a hedgehog protein, e.g., aberrant activation of a hedgehog pathway. The gain-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression of Ci genes, e.g., *Gli1*, *Gli2*, and 5 *Gli3*. The term '*hedgehog* gain-of-function' is also used herein to refer to any similar cellular phenotype (e.g., exhibiting excess proliferation) that occurs due to an alteration anywhere in the hedgehog signal transduction pathway, including, but not limited to, a modification or mutation of *hedgehog* itself. For example, a tumor cell with an abnormally high proliferation rate due to activation of the hedgehog 10 signalling pathway would have a '*hedgehog* gain-of-function' phenotype, even if *hedgehog* is not mutated in that cell.

As used herein, "immortalized cells" refers to cells that have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

15 "Internal epithelial tissue" refers to tissue inside the body that has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

20 The term "IC<sub>50</sub>" means the concentration of a drug which inhibits an activity by 50%, e.g., by reducing the frequency of a condition, such as cell death, by 50%, or by reducing the level of an activity, such as proliferation or a level of biological signalling, by 50%.

25 The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

The term "LD<sub>50</sub>" means the dose of a drug that is lethal in 50% of test subjects.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term "*patched* loss-of-function" refers to an aberrant modification or mutation of a *ptc* gene, or a decreased level of expression of the gene, which results 5 in a phenotype which resembles contacting a cell with a hedgehog protein, e.g., aberrant activation of a hedgehog pathway. The loss-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression or activity of Ci genes, e.g., *Gli1*, *Gli2* and *Gli3*. The term '*ptc* loss-of-function' is also used herein to refer to any similar cellular phenotype (e.g., exhibiting excess proliferation) 10 that occurs due to an alteration anywhere in the hedgehog signal transduction pathway, including, but not limited to, a modification or mutation of *ptc* itself. For example, a tumor cell with an abnormally high proliferation rate due to activation of the hedgehog signalling pathway would have a '*ptc* loss-of-function' phenotype, even if *ptc* is not mutated in that cell.

15 A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., peripheral neuropathy), a disease such as cancer, a syndrome complex such as BCNS, or any other medical condition, is well 20 understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment 25 relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount. Prevention of peripheral neuropathy includes, for example, reducing the number of diagnoses of the infection in a treated population versus an untreated control population, and/or 30 delaying the onset of symptoms of the neuropathy in a treated population versus an untreated control population. Prevention of BCNS includes, for example, reducing

the severity of, or alternatively delaying, the formation of growths in subjects in a treated population versus an untreated control population.

The term "prodrug" is intended to encompass compounds that, under physiological conditions, are converted into the therapeutically active agents of the 5 present invention. A common method for making a prodrug is to include selected moieties that are hydrolyzed under physiological conditions to reveal the desired molecule. In other embodiments, the prodrug is converted by an enzymatic activity of the host animal.

As used herein, "proliferating" and "proliferation" refer to cells undergoing 10 mitosis.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces 15 the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of 20 detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount. Prevention of an infection includes, for example, reducing the number of diagnoses of the infection in a treated population versus an untreated control population, and/or delaying the onset of symptoms of the infection in a treated population versus an untreated 25 control population. Prevention of pain includes, for example, reducing the magnitude of, or alternatively delaying, pain sensations subsequently experienced by subjects in a treated population versus an untreated control population.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of

cutaneous tissue. These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic dermatitis. For example, epidermolytic hyperkeratosis is a form 5 of faulty development of the epidermis. Another example is "epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and bullae either spontaneously or at the site of trauma.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder that alters the skin's regulatory mechanisms. In particular, lesions are 10 formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and 15 polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous 20 glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "*smoothened* gain-of-function" refers to an aberrant modification or mutation of a *smo* gene, or an increased level of expression of the gene, which 25 results in a phenotype that resembles contacting a cell with a hedgehog protein, e.g., aberrant activation of a hedgehog pathway. While not wishing to be bound by any particular theory, it is noted that *ptc* may not signal directly into the cell, but rather modulates the activity of *smoothened*, another membrane-bound protein located downstream of *ptc* in *hedgehog* signaling (Marigo et al., (1996) *Nature* 384: 177-179; Taipale et al. (2002) *Nature* 418, 892-896). The gene *smo* is a segment-polarity 30

gene required for the correct patterning of every segment in *Drosophila* (Alcedo et al., (1996) *Cell* 86: 221-232). Human homologs of *smo* have been identified. See, for example, Stone et al. (1996) *Nature* 384:129-134, and GenBank accession U84401. The *smoothened* gene encodes an integral membrane protein with characteristics of 5 heterotrimeric G-protein-coupled receptors; i.e., 7-transmembrane regions. This protein shows homology to the *Drosophila* *Frizzled* (Fz) protein, a member of the *wingless* pathway. It was originally thought that *smo* encodes a receptor of the Hh signal. However, this suggestion was subsequently disproved, as evidence for *ptc* being the Hh receptor was obtained. Cells that express *Smo* fail to bind Hh, 10 indicating that *smo* does not interact directly with Hh (Nusse, (1996) *Nature* 384: 119-120). Rather, the binding of *Sonic hedgehog* (SHH) to its receptor, *PTCH*, is thought to prevent normal inhibition by *PTCH* of *smoothened* (SMO), a seven-span transmembrane protein.

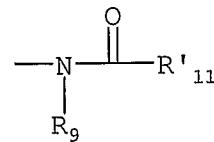
Recently, it has been reported that activating *smoothened* mutations occur in 15 sporadic basal cell carcinoma, Xie et al. (1998) *Nature* 391: 90-2, and primitive neuroectodermal tumors of the central nervous system, Reifenberger et al. (1998) *Cancer Res* 58: 1798-803.

The term "therapeutic index" refers to the therapeutic index of a drug defined as  $LD_{50}/ED_{50}$ .

20 As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

25

The term "acylamino" is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R<sub>9</sub> is as defined above, and R'11 represents a hydrogen, an alkyl, an alkenyl or -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, where m and R<sub>8</sub> are as defined above.

Herein, the term "aliphatic group" refers to a straight-chain, branched-chain, 5 or cyclic aliphatic hydrocarbon group and includes saturated and unsaturated aliphatic groups, such as an alkyl group, an alkenyl group, and an alkynyl group.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

10 The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxy groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxy, such as can be 15 represented by one of -O-alkyl, -O-alkenyl, -O-alkynyl, -O-(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, where m and R<sub>8</sub> are described above.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. 20 In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C<sub>1</sub>-C<sub>30</sub> for straight chains, C<sub>3</sub>-C<sub>30</sub> for branched chains), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

25 Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having

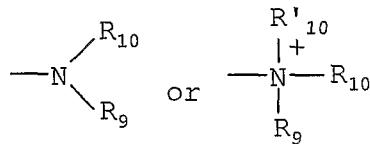
substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxy carbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxy, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulphydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF<sub>3</sub>, -CN and the like.

Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxy, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF<sub>3</sub>, -CN, and the like.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Throughout the application, preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, -S-alkynyl, and -S-(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, wherein m and R<sub>8</sub> are defined above. Representative alkylthio groups include methylthio, ethylthio, and the like.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:

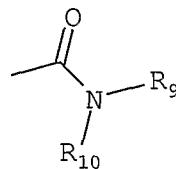


5    wherein R<sub>9</sub>, R<sub>10</sub> and R'<sub>10</sub> each independently represent a hydrogen, an alkyl, an alkenyl, -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, or R<sub>9</sub> and R<sub>10</sub> taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R<sub>8</sub> represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of

10   R<sub>9</sub> or R<sub>10</sub> can be a carbonyl, e.g., R<sub>9</sub>, R<sub>10</sub> and the nitrogen together do not form an imide. In certain such embodiments, neither R<sub>9</sub> and R<sub>10</sub> is attached to N by a carbonyl, e.g., the amine is not an amide or imide, and the amine is preferably basic, e.g., its conjugate acid has a pK<sub>a</sub> above 7. In even more preferred embodiments, R<sub>9</sub> and R<sub>10</sub> (and optionally R'<sub>10</sub>) each independently represent a hydrogen, an alkyl, an alkenyl, or -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R<sub>9</sub> and R<sub>10</sub> is an alkyl group.

15   R<sub>9</sub> or R<sub>10</sub> can be a carbonyl, e.g., R<sub>9</sub>, R<sub>10</sub> and the nitrogen together do not form an imide. In certain such embodiments, neither R<sub>9</sub> and R<sub>10</sub> is attached to N by a carbonyl, e.g., the amine is not an amide or imide, and the amine is preferably basic, e.g., its conjugate acid has a pK<sub>a</sub> above 7. In even more preferred embodiments, R<sub>9</sub> and R<sub>10</sub> (and optionally R'<sub>10</sub>) each independently represent a hydrogen, an alkyl, an alkenyl, or -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R<sub>9</sub> and R<sub>10</sub> is an alkyl group.

The term "amido" is art-recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



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wherein R<sub>9</sub>, R<sub>10</sub> are as defined above. Preferred embodiments of the amide will not include imides that may be unstable.

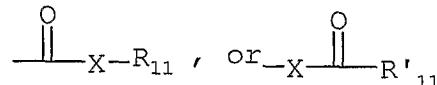
The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The term "aryl" as used herein includes 5-, 6-, and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having 5 heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxy, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, 10 alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, -CF<sub>3</sub>, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, 15 cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

The term "carbonyl" is art-recognized and includes such moieties as can be represented by the general formula:

20



wherein X is a bond or represents an oxygen or a sulfur, and R<sub>11</sub> represents a hydrogen, an alkyl, an alkenyl, -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub> or a pharmaceutically acceptable salt, R'<sub>11</sub> represents a hydrogen, an alkyl, an alkenyl or -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, where m and R<sub>8</sub> are as defined above. Where X is an oxygen and R<sub>11</sub> or R'<sub>11</sub> is not hydrogen, the 25 formula represents an "ester". Where X is an oxygen, and R<sub>11</sub> is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R<sub>11</sub> is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'<sub>11</sub> is hydrogen, the formula represents a "formate". In general, where the oxygen

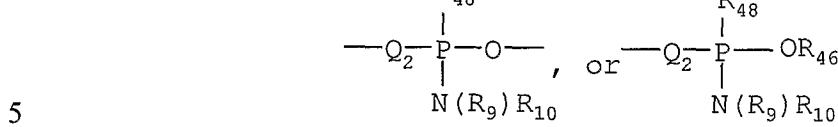
atom of the above formula is replaced by sulfur, the formula represents a "thiocarbonyl" group. Where X is a sulfur and R<sub>11</sub> or R'<sub>11</sub> is not hydrogen, the formula represents a "thioester." Where X is a sulfur and R<sub>11</sub> is hydrogen, the formula represents a "thiocarboxylic acid." Where X is a sulfur and R<sub>11</sub>' is 5 hydrogen, the formula represents a "thiolformate." On the other hand, where X is a bond, and R<sub>11</sub> is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R<sub>11</sub> is hydrogen, the above formula represents an "aldehyde" group.

10 The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

15 The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, 20 quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, 25 aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF<sub>3</sub>, -CN, or the like.

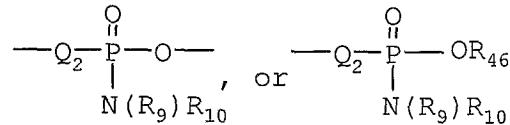
As used herein, the term "nitro" means  $-NO_2$ ; the term "halogen" designates  $-F$ ,  $-Cl$ ,  $-Br$  or  $-I$ ; the term "sulphydryl" means  $-SH$ ; the term "hydroxyl" means  $-OH$ ; and the term "sulfonyl" means  $-SO_2-$ .

A "phosphonamidite" can be represented in the general formula:



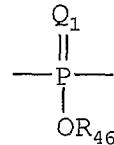
wherein  $R_9$  and  $R_{10}$  are as defined above,  $Q_2$  represents O, S or N, and  $R_{48}$  represents a lower alkyl or an aryl,  $Q_2$  represents O, S or N.

A "phosphoramidite" can be represented in the general formula:

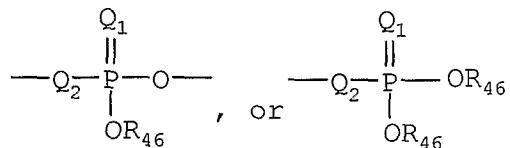


10 wherein  $R_9$  and  $R_{10}$  are as defined above, and  $Q_2$  represents O, S or N.

A "phosphoryl" can in general be represented by the formula:



wherein  $Q_1$  represented S or O, and  $R_{46}$  represents hydrogen, a lower alkyl or an aryl. When used to substitute, for example, an alkyl, the phosphoryl group of the 15 phosphorylalkyl can be represented by the general formula:



wherein  $Q_1$  represented S or O, and each  $R_{46}$  independently represents hydrogen, a lower alkyl or an aryl,  $Q_2$  represents O, S or N. When  $Q_1$  is an S, the phosphoryl moiety is a "phosphorothioate".

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocycls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each 5 of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF<sub>3</sub>, -CN, or the like.

10 The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, 15 T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2<sup>nd</sup> ed.; Wiley: New York, 1991).

A "selenoalkyl" refers to an alkyl group having a substituted seleno group attached thereto. Exemplary "selenoethers" which may be substituted on the alkyl are selected from one of -Se-alkyl, -Se-alkenyl, -Se-alkynyl, and -Se-(CH<sub>2</sub>)<sub>m</sub>-R<sub>g</sub>, m and 20 R<sub>g</sub> being defined above.

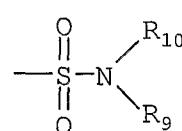
As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. 25 Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. This

invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the 5 substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

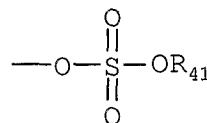
The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:

10



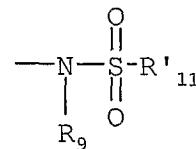
in which R<sub>9</sub> and R<sub>10</sub> are as defined above.

The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



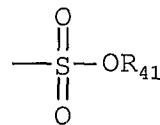
15 in which R<sub>41</sub> is as defined above.

The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



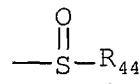
in which R<sub>9</sub> and R'<sub>11</sub> are as defined above.

20 The term "sulfonate" is art-recognized and includes a moiety that can be represented by the general formula: :



in which R<sub>41</sub> is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The terms "sulfoxido" or "sulfinyl", as used herein, refers to a moiety that can be represented by the general formula:



in which R<sub>44</sub> is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls, 10 iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

As used herein, the definition of each expression, e.g., alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

15 The terms triflyl, tosyl, mesyl, and nonaflyl are art-recognized and refer to trifluoromethanesulfonyl, *p*-toluenesulfonyl, methanesulfonyl, and nonafluorobutanesulfonyl groups, respectively. The terms triflate, tosylate, mesylate, and nonaflate are art-recognized and refer to trifluoromethanesulfonate ester, *p*-toluenesulfonate ester, methanesulfonate ester, and nonafluorobutanesulfonate ester 20 functional groups and molecules that contain said groups, respectively.

The abbreviations Me, Et, Ph, Tf, Nf, Ts, Ms represent methyl, ethyl, phenyl, trifluoromethanesulfonyl, nonafluorobutanesulfonyl, *p*-toluenesulfonyl and methanesulfonyl, respectively. A more comprehensive list of the abbreviations utilized by organic chemists of ordinary skill in the art appears in the first issue of 25 each volume of the *Journal of Organic Chemistry*; this list is typically presented in a table entitled Standard List of Abbreviations. The abbreviations contained in said

list, and all abbreviations utilized by organic chemists of ordinary skill in the art are hereby incorporated by reference.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, 5 including cis- and trans-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

10 If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic 15 functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

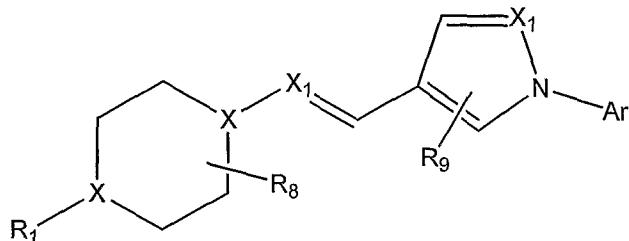
Contemplated equivalents of the compounds described above include 20 compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g., the ability to inhibit hedgehog signaling), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for 25 example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. In these reactions, it is also possible to make use of variants which are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in 30 accordance with the Periodic Table of the Elements, CAS version, Handbook of

Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and unbranched, 5 carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

*III. Exemplary Compounds of the Invention.*

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be 10 readily identified, for example, by such drug screening assays as described herein. For example, compounds useful in the subject methods include compounds may be represented by general formula (I):



Formula I

15 wherein, as valence and stability permit,

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring;

X represents, independently for each occurrence, N or CR', preferably N;

X<sub>1</sub> represents, independently for each occurrence, N or CR'', preferably N;

20 R' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate, preferably H, halogen, alkyl, hydroxyl, alkoxy, or amino;

R'' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate, preferably H, halogen, alkyl, hydroxyl, alkoxy, or amino;

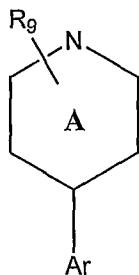
5 R<sub>1</sub> represents a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heterocyclyl alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl, preferably alkyl, heteroaralkyl, or aralkyl (such as substituted or unsubstituted benzyl or phenethyl);

10 R<sub>8</sub> represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, =O, =S, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, preferably lower alkyl or 0 substituents; and

15 R<sub>9</sub> represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, preferably halogen, nitro, cyano, or lower alkyl, or 0 substituents, or a pharmaceutically acceptable salt thereof.

20 Exemplary compounds of Formula I are shown in Scheme 1 below. In certain embodiments, compounds of Formula I of the invention do not include the *o*-chlorobenzyl substituted compound of Scheme 1.

25 In still other embodiments, the invention provides antagonists having a structure of Formula II:

**Formula II**

wherein, as valence and stability permit,

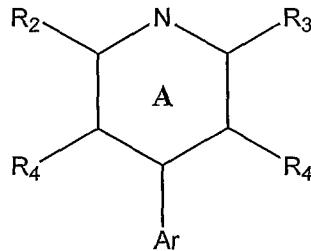
A represents a pyridyl or dihydropyridyl ring;

5 Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring; and

R<sub>9</sub> represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, 10 phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, preferably H, halogen, alkyl, hydroxyl, alkoxy, thioether, acyl, alkylamino, or amino, or a pharmaceutically acceptable salt thereof.

15 Exemplary compounds of Formula II are shown in Scheme 3 below. In certain embodiments, compounds of Formula II of the invention do not include the methoxyphenyl-substituted compound at the top center of Scheme 3.

In yet other embodiments, the invention provides compounds having a structure of Formula III:



20

### Formula III

wherein, independently for each occurrence,

A represents a pyridyl or dihydropyridyl ring;

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a

5 substituted or unsubstituted phenyl ring; and

$R_2$  represents a thioether, preferably substituted with an amido, ester, carboxyl, or

acyl, such as substituted or unsubstituted benzoyl or aminocarbonyl;

$R_3$  represents H, alkyl, alkenyl, alkynyl, or cycloalkyl; and

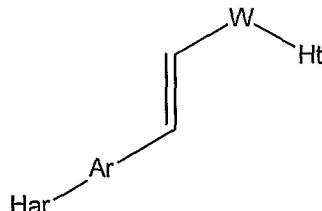
R<sub>4</sub> represents a substituent selected from H, halogen, alkyl, alkenyl, alkynyl, aryl,

10 hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, preferably H, halogen, alkyl, alkenyl, alkynyl, hydroxyl, nitro, cyano, or acyl (e.g., one occurrence of cyano and one occurrence of acyl, such as acetyl or other alkylcarbonyl),  
15

Exemplary compounds of Formula III are shown in Scheme 3 below. In certain embodiments, compounds of Formula III of the invention do not include the methoxyphenyl-substituted compound at the top center of Scheme 3. In preferred

20 compounds of Formulae II and III, when A represents a dihydropyridyl ring, the nitrogen atom and the Ar-bearing carbon atom bear hydrogen substituents. In certain such embodiments, the dihydropyridyl ring bears an acyl, ester, carboxyl, carboxamide, or other carbonyl substituent, e.g., adjacent (vicinal) to the Ar substituent.

25 In additional embodiments, the present invention provides compounds having a structure of Formula IV:



Formula IV

wherein, as valence and stability permit,

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring;

5 W represents C=O, C=S, or SO<sub>2</sub>;

Har represents a substituted or unsubstituted heteroaryl ring, preferably a substituted or unsubstituted pyridyl ring, such as 2-pyridyl, preferably substituted with a C<sub>1</sub>-C<sub>10</sub> alkyl, such as an unbranched alkyl at the 5-position of the pyridine ring;

10 Ht represents OR or NR'''<sub>2</sub>, such as a mono- or di-substituted amino group, e.g., a dialkylamino or a cyclic amino group such as a piperidyl, piperazinyl, or morpholino group;

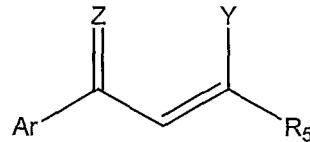
R represents H or lower alkyl; and

R''' represents H, lower alkyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heterocyclyl

15 alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl, preferably aryl or aralkyl, or a pharmaceutically acceptable salt thereof.

Exemplary compounds of Formula IV are shown in Scheme 4 below. In certain embodiments, compounds of Formula IV of the invention do not include the pentylpyridine-piperidinamide compound (200 nM) of Scheme 4.

20 In still another embodiment, the present invention provides compounds having a structure of Formula V:

Formula V

wherein, as valence and stability permit,

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring, such as an ortho-hydroxyphenyl group optionally substituted with one or more halogen, hydroxy, alkoxy, amino, alkylamino, alkyl, thiol, alkylthio, ester, amido, carboxyl, nitro, or cyano substituents;

5 Y represents OH or NR<sub>2</sub>, preferably NH<sub>2</sub>;

Z represents O or S;

R represents H or lower alkyl; and

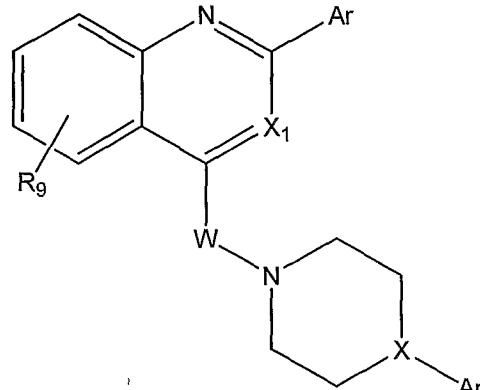
R<sub>5</sub> represents substituted or unsubstituted lower alkyl, preferably a trihalomethyl group,

10 or a pharmaceutically acceptable salt thereof.

Exemplary compounds of Formula V are shown in Scheme 5 below.

In still yet another embodiment, the invention provides compounds having a structure of Formula VI:

15



Formula VI

wherein, as valence and stability permit,

Ar, independently for each occurrence, represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring;

20 W represents C=O, C=S, or SO<sub>2</sub>;

X represents, independently for each occurrence, N or CR', preferably N;

X<sub>1</sub> represents, independently for each occurrence, N or CR'', preferably CR;

R represents H or lower alkyl; and

R' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl,

aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol,

amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide,

5 anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl,

aldehyde, ester, carbamate, or carbonate, preferably H, halogen, alkyl,

hydroxyl, alkoxy, or amino;

R'' represents, independently for each occurrence, H, halogen, alkyl, alkenyl,

alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano,

10 thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl,

carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl,

selenoether, acyl, aldehyde, ester, carbamate, or carbonate, preferably H,

halogen, alkyl, hydroxyl, alkoxy, or amino; and

R<sub>9</sub> represents from 0-4 substituents on the ring to which it is attached, independently

15 selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy,

silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido,

phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether,

alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and

carbonate, preferably from halogen, alkyl, cyano, and nitro,

20 or a pharmaceutically acceptable salt thereof.

Exemplary compounds of Formula VI are shown in Scheme 6 below.

Because compounds having structures of Formulae V and VI appear to act

downstream of *smoothened*, these compounds can be used to inhibit the *hedgehog* pathway in cells that have a *smoothened* gain-of-function mutation that renders them

25 at least partially resistant to inhibitors that act directly upon *hedgehog*, *patched*, or

*smoothened*. Accordingly, the present invention contemplates *hedgehog* antagonists

that inhibit the *hedgehog* pathway in a *smoothened* gain-of-function cell with an IC<sub>50</sub>

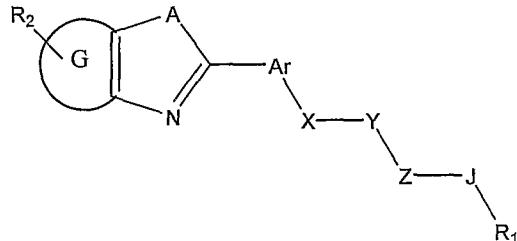
within a factor of 5, or even a factor of 2 or 1, of the IC<sub>50</sub> with which they inhibit the

*hedgehog* pathway in a *patched*-null cell. Such antagonists are suitable not only for

30 normal cells, or cells with aberrant activity or expression of *hedgehog*, *patched*, or

*smoothened*, but also for cells with defects in the *hedgehog* pathway downstream of these proteins.

In certain embodiments, an antagonist has the structure of Formula VII:



5

**Formula VII**

wherein, as valence and stability permit,

X and Z, independently, represent -N(R<sub>7</sub>)-, -O-, -S-, -(R<sub>7</sub>)N-N(R<sub>7</sub>)-, -ON(R<sub>7</sub>)-, or a direct bond, preferably -N(R<sub>7</sub>)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR<sub>7</sub>)-, SO<sub>2</sub>, or SO, preferably -C(=O)-, 10 SO<sub>2</sub>, or -C(=S)-;

A represents O, S, or NR<sub>7</sub>, preferably O or NH, and most preferably NH;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring.

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, such as a 15 substituted or unsubstituted phenyl ring;

R<sub>1</sub> represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

R<sub>2</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, 20 carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate), ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R<sub>8</sub>, J-OH, J-lower alkyl, J-lower alkenyl, J-R<sub>8</sub>, J-SH, J-NH<sub>2</sub>, protected forms of the above, or any two R<sub>2</sub>, when occurring more than once in a

cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

R<sub>7</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-  
5 heterocyclyl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

R<sub>8</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocyclyl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and  
10

J represents, independently for each occurrence, a chain having from 0-8 (preferably from 0-4) units selected from CK<sub>2</sub>, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl.

15 In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R<sub>8</sub>)-, -O-, -S-, and preferably represents NH.

20 In certain embodiments, R<sub>1</sub> includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

In certain embodiments, X and the ring comprising A are disposed on Ar in a meta (i.e., 1,3) relationship.

In certain embodiments, G represents a phenyl or piperidine ring.

25 In certain embodiments, J is absent.

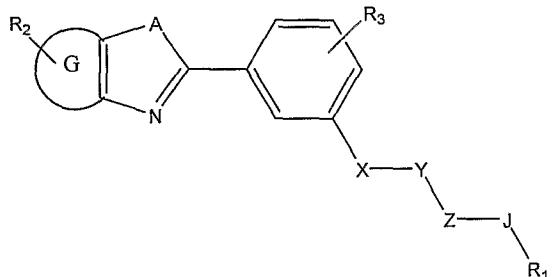
In certain embodiments, R<sub>2</sub> represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), a substituted

or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

In certain embodiments, the subject compound is selected from the compounds depicted in Figure 32.

5

In certain embodiments, compounds useful in the present invention may be represented by Formula VIIa:



**Formula VIIa**

10 wherein, as valence and stability permit,

X and Z, independently, represent -N(R<sub>7</sub>)-, -O-, -S-, -(R<sub>7</sub>)N-N(R<sub>7</sub>)-, -ON(R<sub>7</sub>)-, or a direct bond, preferably -N(R<sub>7</sub>)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR<sub>7</sub>)-, SO<sub>2</sub>, or SO, preferably -C(=O)-, SO<sub>2</sub>, or -C(=S)-;

15 A represents O, S, or NR<sub>7</sub>, preferably O or NH, and most preferably NH;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring.

R<sub>1</sub> represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

20 R<sub>2</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate), ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate,

phosphinate, J-R<sub>8</sub>, J-OH, J-lower alkyl, J-lower alkenyl, J-R<sub>8</sub>, J-SH, J-NH<sub>2</sub>, protected forms of the above, or any two R<sub>2</sub>, when occurring more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

5 R<sub>3</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, hydroxyl, alkoxy, amino, alkylamino, cyano, nitro, substituted or unsubstituted lower alkyl, and acyl, preferably halogen, lower alkoxy, or substituted or unsubstituted lower alkyl;

10 R<sub>7</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-heterocyclyl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

15 R<sub>8</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocyclyl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and

J represents, independently for each occurrence, a chain having from 0-8 (preferably from 0-4) units selected from CK<sub>2</sub>, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl.

20

In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R<sub>8</sub>)-, -O-, -S-, and preferably represents NH.

25 In certain embodiments, R<sub>1</sub> includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

In certain embodiments, G represents a phenyl or piperidine ring.

In certain embodiments, J is absent.

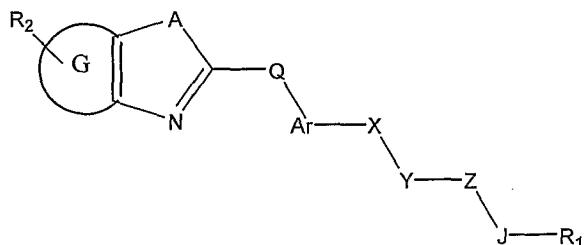
In certain embodiments, R<sub>2</sub> represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

5 In certain embodiments, R<sub>3</sub> includes a substituent, such as a substituted or unsubstituted alkyl or a halogen, at a position para either to X or to the ring including A.

In certain embodiments, the subject compound is selected from the compounds depicted in Figure 32.

10

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be readily identified, for example, by such drug screening assays as described herein. For example, compounds useful in the subject methods include compounds may be 15 represented by Formula VIIb:



**Formula VIIb**

wherein, as valence and stability permit,

20 X and Z, independently, represent -N(R<sub>7</sub>)-, -O-, -S-, -(R<sub>7</sub>)N-N(R<sub>7</sub>)-, -ON(R<sub>7</sub>)-, or a direct bond, preferably -N(R<sub>7</sub>)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR<sub>7</sub>)-, SO<sub>2</sub>, or SO, preferably -C(=O)-, SO<sub>2</sub>, or -C(=S)-;

A represents O, S, or NR<sub>7</sub>, preferably O or NH, and most preferably NH;

25 G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring;

Q is absent, or represents CK<sub>2</sub>, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl;

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, such as a substituted or unsubstituted phenyl ring;

5 R<sub>1</sub> represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocycl, or cycloalkyl, including polycyclic groups;

R<sub>2</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate),  
10 ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R<sub>8</sub>, J-OH, J-lower alkyl, J-lower alkenyl, J-R<sub>8</sub>, J-SH, J-NH<sub>2</sub>, protected forms of the above, or any two R<sub>2</sub>, when occurring more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered  
15 cycloalkyl, aryl, or heteroaryl;

R<sub>7</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-heterocycl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

20 R<sub>8</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocycl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and

J represents, independently for each occurrence, a chain having from 0-8  
25 (preferably from 0-4) units selected from CK<sub>2</sub>, NK, O, and S.

In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R<sub>8</sub>)-, -O-, -S-, and preferably represents NH.

In certain embodiments, R<sub>1</sub> includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

5 In certain embodiments, X and the ring comprising A are disposed on Ar in a meta (i.e., 1,3) relationship.

In certain embodiments, G represents a phenyl or piperidine ring.

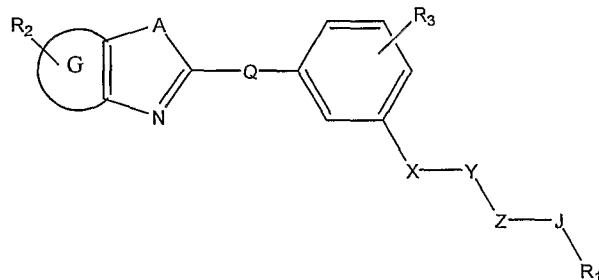
In certain embodiments, J is absent.

10 In certain embodiments, R<sub>2</sub> represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

In certain embodiments, the subject compound is selected from the compounds depicted in Figure 32.

15

In certain embodiments, compounds useful in the present invention may be represented by Formula VIIc:



**Formula VIIc**

20 wherein, as valence and stability permit,

X and Z, independently, represent -N(R<sub>7</sub>)-, -O-, -S-, -(R<sub>7</sub>)N-N(R<sub>7</sub>)-, -ON(R<sub>7</sub>)-, or a direct bond, preferably -N(R<sub>7</sub>)-, -O-, -S-, or a direct bond;

Y represents -C(=O)-, -C(=S)-, -C(=NR<sub>7</sub>)-, SO<sub>2</sub>, or SO, preferably -C(=O)-, SO<sub>2</sub>, or -C(=S)-;

A represents O, S, or NR<sub>7</sub>, preferably O or NH, and most preferably NH;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached, preferably an aryl or heteroaryl ring.

Q is absent, or represents CK<sub>2</sub>, NK, O, and S, wherein K represents, 5 independently for each occurrence, H or lower alkyl;

R<sub>1</sub> represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

R<sub>2</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group (e.g., ester, 10 carboxyl, or formyl), thiocarbonyl (e.g., thioester, thiocarboxylate, or thioformate), ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R<sub>8</sub>, J-OH, J-lower alkyl, J-lower alkenyl, J-R<sub>8</sub>, J-SH, J-NH<sub>2</sub>, 15 protected forms of the above, or any two R<sub>2</sub>, when occurring more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

R<sub>3</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, hydroxyl, alkoxy, amino, alkylamino, cyano, nitro, substituted or unsubstituted lower alkyl, and acyl, preferably halogen, lower alkoxy, or substituted 20 or unsubstituted lower alkyl;

R<sub>7</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), J-cycloalkyl (e.g., substituted or unsubstituted), J-heterocyclyl (e.g., substituted or unsubstituted), J-aryl (e.g., substituted or unsubstituted), J-heteroaryl (e.g., substituted or unsubstituted);

25 R<sub>8</sub>, independently for each occurrence, represents H, lower alkyl (e.g., substituted or unsubstituted), cycloalkyl (e.g., substituted or unsubstituted), heterocyclyl (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), or heteroaryl (e.g., substituted or unsubstituted); and

J represents, independently for each occurrence, a chain having from 0-8 (preferably from 0-4) units selected from CK<sub>2</sub>, NK, O, and S.

5 In certain embodiments, at least one of Z and X is not a direct bond. In certain embodiments, X-Y-Z includes an amide, urea, or sulfonamide. In certain embodiments, X is selected from -N(R<sub>8</sub>)-, -O-, -S-, and preferably represents NH.

10 In certain embodiments, R<sub>1</sub> includes an aryl or heteroaryl ring, optionally substituted with from 1-5 substituents, such as nitro, halogen, cyano, lower alkyl, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), alkoxy, alkylamino, a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to the aryl or heteroaryl ring.

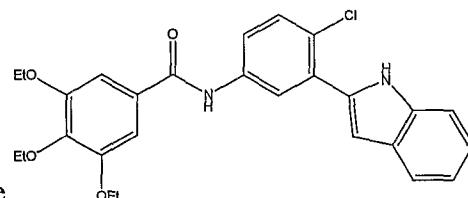
In certain embodiments, G represents a phenyl or piperidine ring.

In certain embodiments, J is absent.

15 In certain embodiments, R<sub>2</sub> represents from 1-4 substituents selected from halogen, cyano, nitro, alkoxy, amino, acylamino (e.g., R<sub>8</sub>-C(=O)NH-), a substituted or unsubstituted cycloalkyl, heterocyclyl, aryl, or heteroaryl fused to G, and substituted or unsubstituted lower alkyl.

In certain embodiments, R<sub>3</sub> includes a substituent, such as a substituted or unsubstituted alkyl or a halogen, at a position para either to X or to the ring including A.

20



Compounds of Formula VII include

25 In certain embodiments, the subject antagonists can be chosen on the basis of their selectively for the *hedgehog* pathway. This selectivity can be for the *hedgehog*

pathway versus other pathways, or for selectivity between particular *hedgehog* pathways, e.g., *ptc-1*, *ptc-2*, etc.

In certain preferred embodiments, the subject inhibitors inhibit *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function mediated signal transduction with an ED<sub>50</sub> of 1 mM or less, more preferably of 1  $\mu$ M or less, and even more preferably of 1 nM or less. Similarly, in certain preferred embodiments, the subject inhibitors inhibit activity of the *hedgehog* pathway with a K<sub>i</sub> less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

10 In particular embodiments, the small molecule is chosen for use because it is more selective for one patched isoform over the next, e.g., 10-fold, and more preferably at least 100- or even 1000-fold more selective for one *patched* pathway (*ptc-1*, *ptc-2*) over another.

15 In certain embodiments, a compound which is an antagonist of the *hedgehog* pathway is chosen to selectively antagonize *hedgehog* activity over protein kinases other than PKA, such as PKC, e.g., the compound modulates the activity of the *hedgehog* pathway at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly.

20 Thus, for example, a preferred inhibitor of the *hedgehog* pathway may inhibit *hedgehog* activity with a K<sub>i</sub> at least an order of magnitude lower than its K<sub>i</sub> for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, the K<sub>i</sub> for PKA inhibition is less than 10 nM, preferably less than 1 nM, even more 25 preferably less than 0.1 nM.

#### *IV. Exemplary Applications of Method and Compositions*

Another aspect of the present invention relates to a method of modulating a differentiated state, survival, and/or proliferation of a cell having a *ptc* loss-of-

function, *hedgehog* gain-of-function, or *smoothened* gain-of-function, by contacting the cells with a *hedgehog* antagonist according to the subject method and as the circumstances may warrant.

For instance, it is contemplated by the invention that, in light of the findings 5 of an apparently broad involvement of *hedgehog*, *ptc*, and *smoothened* in the formation of ordered spatial arrangements of differentiated tissues in vertebrates, the subject method could be used as part of a process for generating and/or maintaining an array of different vertebrate tissue both *in vitro* and *in vivo*. The *hedgehog* antagonist, whether inductive or anti-inductive with respect proliferation or 10 differentiation of a given tissue, can be, as appropriate, any of the preparations described above.

For example, the present method is applicable to cell culture techniques wherein, whether for genetic or biochemical reasons, the cells have a *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function phenotype. *In* 15 *vitro* neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors such as nerve growth factor (NGF), ciliary trophic factors (CNTF), and brain derived neurotrophic factor (BDNF). One use of the present method may be in cultures of neuronal stem cells, such as in the use of such cultures 20 for the generation of new neurons and glia. In such embodiments of the subject method, the cultured cells can be contacted with a *hedgehog* antagonist of the present invention in order to alter the rate of proliferation of neuronal stem cells in the culture and/or alter the rate of differentiation, or to maintain the integrity of a culture of certain terminally differentiated neuronal cells. In an exemplary 25 embodiment, the subject method can be used to culture, for example, sensory neurons or, alternatively, motorneurons. Such neuronal cultures can be used as convenient assay systems as well as sources of implantable cells for therapeutic treatments.

According to the present invention, large numbers of non-tumorigenic neural 30 progenitor cells can be perpetuated *in vitro* and their rate of proliferation and/or

differentiation can be affected by contact with *hedgehog* antagonists of the present invention. Generally, a method is provided comprising the steps of isolating neural progenitor cells from an animal, perpetuating these cells *in vitro* or *in vivo*, preferably in the presence of growth factors, and regulating the differentiation of 5 these cells into particular neural phenotypes, e.g., neurons and glia, by contacting the cells with a *hedgehog* antagonist.

Progenitor cells are thought to be under a tonic inhibitory influence that maintains the progenitors in a suppressed state until their differentiation is required. However, recent techniques have been provided which permit these cells to be 10 proliferated, and unlike neurons that are terminally differentiated and therefore non-dividing, they can be produced in unlimited number and are highly suitable for transplantation into heterologous and autologous hosts with neurodegenerative diseases.

By "progenitor" it is meant an oligopotent or multipotent stem cell that is 15 able to divide without limit and, under specific conditions, can produce daughter cells that terminally differentiate such as into neurons and glia. These cells can be used for transplantation into a heterologous or autologous host. By heterologous is meant a host other than the animal from which the progenitor cells were originally derived. By autologous is meant the identical host from which the cells were 20 originally derived.

Cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue from any animal. By any animal is meant any multicellular animal that contains nervous tissue. More particularly, is meant any fish, reptile, bird, amphibian or mammal and the like. The most preferable donors are mammals, especially mice 25 and humans.

In the case of a heterologous donor animal, the animal may be euthanized, and the brain and specific area of interest removed using a sterile procedure. Brain areas of particular interest include any area from which progenitor cells can be obtained which will serve to restore function to a degenerated area of the host's 30 brain. These regions include areas of the central nervous system (CNS) including the

cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the peripheral nervous system (PNS) including the carotid body and the adrenal medulla. More particularly, these areas include regions in the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell 5 groups such as the globus pallidus, the subthalamic nucleus, the nucleus basalis which is found to be degenerated in Alzheimer's Disease patients, or the substantia nigra pars compacta which is found to be degenerated in Parkinson's Disease patients.

Human heterologous neural progenitor cells may be derived from fetal tissue 10 obtained from elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, in particular during epilepsy surgery, and more particularly during temporal lobectomies and hippocampal resections.

15 Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Dissociation can be obtained using any known procedure, including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument or by mincing with a scalpel to allow outgrowth of specific cell 20 types from a tissue. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, and 10 mM D-glucose. Low Ca<sup>2+</sup> aCSF contains the same ingredients except for MgCl<sub>2</sub> at a concentration of 3.2 25 mM and CaCl<sub>2</sub> at a concentration of 0.1 mM.

Dissociated cells can be placed into any known culture medium capable of supporting cell growth, including MEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as

transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. In some cases, the medium may contain serum derived from bovine, equine, chicken and the like. A particularly preferable medium for cells is a 5 mixture of DMEM and F-12.

Conditions for culturing should be close to physiological conditions. The pH of the culture media should be close to physiological pH, preferably between pH 6-8, more preferably close to pH 7, even more particularly about pH 7.4. Cells should be cultured at a temperature close to physiological temperature, preferably between 30 10 °C-40 °C, more preferably between 32 °C-38 °C, and most preferably between 35 °C-37 °C.

Cells can be grown in suspension or on a fixed substrate, but proliferation of the progenitors is preferably done in suspension to generate large numbers of cells by formation of "neurospheres" (see, for example, Reynolds et al. (1992) *Science* 15 255:1070-1709; and PCT Publications WO93/01275, WO94/09119, WO94/10292, and WO94/16718). In the case of propagating (or splitting) suspension cells, flasks are shaken well and the neurospheres allowed to settle on the bottom corner of the flask. The spheres are then transferred to a 50 ml centrifuge tube and centrifuged at low speed. The medium is aspirated, the cells resuspended in a small amount of 20 medium with growth factor, and the cells mechanically dissociated and resuspended in separate aliquots of media.

Cell suspensions in culture medium are supplemented with any growth factor which allows for the proliferation of progenitor cells and seeded in any receptacle capable of sustaining cells, though as set out above, preferably in culture flasks or 25 roller bottles. Cells typically proliferate within 3-4 days in a 37 °C incubator, and proliferation can be reinitiated at any time after that by dissociation of the cells and resuspension in fresh medium containing growth factors.

In the absence of substrate, cells lift off the floor of the flask and continue to proliferate in suspension forming a hollow sphere of undifferentiated cells. After 30 approximately 3-10 days *in vitro*, the proliferating clusters (neurospheres) are fed

every 2-7 days, and more particularly every 2-4 days by gentle centrifugation and resuspension in medium containing growth factor.

After 6-7 days *in vitro*, individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, more 5 particularly by triturating the neurospheres with a pipette. Single cells from the dissociated neurospheres are suspended in culture medium containing growth factors, and differentiation of the cells can be controlled in culture by plating (or resuspending) the cells in the presence of a *hedgehog* antagonist.

To further illustrate other uses of the subject *hedgehog* antagonists, it is 10 noted that intracerebral grafting has emerged as an additional approach to central nervous system therapies. For example, one approach to repairing damaged brain tissues involves the transplantation of cells from fetal or neonatal animals into the adult brain (Dunnett et al. (1987) *J Exp Biol* 123:265-289; and Freund et al. (1985) *J Neurosci* 5:603-616). Fetal neurons from a variety of brain regions can be 15 successfully incorporated into the adult brain, and such grafts can alleviate behavioral defects. For example, movement disorder induced by lesions of dopaminergic projections to the basal ganglia can be prevented by grafts of embryonic dopaminergic neurons. Complex cognitive functions that are impaired after lesions of the neocortex can also be partially restored by grafts of embryonic 20 cortical cells. The subject method can be used to regulate the growth state in the culture, or where fetal tissue is used, especially neuronal stem cells, can be used to regulate the rate of differentiation of the stem cells.

Stem cells useful in the present invention are generally known. For example, 25 several neural crest cells have been identified, some of which are multipotent and likely represent uncommitted neural crest cells, and others of which can generate only one type of cell, such as sensory neurons, and likely represent committed progenitor cells. The role of *hedgehog* antagonists employed in the present method to culture such stem cells can be to regulate differentiation of the uncommitted progenitor, or to regulate further restriction of the developmental fate of a committed 30 progenitor cell towards becoming a terminally differentiated neuronal cell. For

example, the present method can be used *in vitro* to regulate the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The *hedgehog* antagonists can be used alone, or can be used in combination 5 with other neurotrophic factors that act to more particularly enhance a particular differentiation fate of the neuronal progenitor cell.

In addition to the implantation of cells cultured in the presence of the subject *hedgehog* antagonists, yet another aspect of the present invention concerns the therapeutic application of a *hedgehog* antagonist to regulate the growth state of 10 neurons and other neuronal cells in both the central nervous system and the peripheral nervous system. The ability of *ptc*, *hedgehog*, and *smoothened* to regulate neuronal differentiation during development of the nervous system and also presumably in the adult state indicates that, in certain instances, the subject *hedgehog* antagonists can be expected to facilitate control of adult neurons with 15 regard to maintenance, functional performance, and aging of normal cells; repair and regeneration processes in chemically or mechanically lesioned cells; and treatment of degeneration in certain pathological conditions. In light of this understanding, the present invention specifically contemplates applications of the subject method to the treatment protocol of (prevention and/or reduction of the severity of) neurological 20 conditions deriving from: (i) acute, subacute, or chronic injury to the nervous system, including traumatic injury, chemical injury, vascular injury and deficits (such as the ischemia resulting from stroke), together with infectious/inflammatory and tumor-induced injury; (ii) aging of the nervous system including Alzheimer's disease; (iii) chronic neurodegenerative diseases of the nervous system, including 25 Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations; and (iv) chronic immunological diseases of the nervous system or affecting the nervous system, including multiple sclerosis.

As appropriate, the subject method can also be used in generating nerve prostheses for the repair of central and peripheral nerve damage. In particular, where 30 a crushed or severed axon is intubulated by use of a prosthetic device, *hedgehog*

antagonists can be added to the prosthetic device to regulate the rate of growth and regeneration of the dendritic processes. Exemplary nerve guidance channels are described in U.S. patents 5,092,871 and 4,955,892.

In another embodiment, the subject method can be used in the treatment of  
5 neoplastic or hyperplastic transformations such as may occur in the central nervous system. For instance, the *hedgehog* antagonists can be utilized to cause such transformed cells to become either post-mitotic or apoptotic. The present method may, therefore, be used as part of a treatment for, e.g., malignant gliomas, meningiomas, medulloblastomas, neuroectodermal tumors, and ependymomas.

10 In a preferred embodiment, the subject method can be used as part of a treatment regimen for malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors.

In certain embodiments, the subject method is used as part of treatment  
program for medulloblastoma. Medulloblastoma, a primary brain tumor, is the most  
15 common brain tumor in children. A medulloblastoma is a primitive neuroectodermal tumor arising in the posterior fossa. They account for approximately 25% of all pediatric brain tumors (Miller). Histologically, they are small round cell tumors commonly arranged in true rosettes, but may display some differentiation to astrocytes, ependymal cells or neurons (Rorke; Kleihues). PNET's may arise in other  
20 areas of the brain including the pineal gland (pineoblastoma) and cerebrum. Those arising in the supratentorial region generally fare worse than their PF counterparts.

Medulloblastoma/PNET's are known to recur anywhere in the CNS after resection, and can even metastasize to bone. Pretreatment evaluation should therefore include an examination of the spinal cord to exclude the possibility of  
25 "dropped metastases". Gadolinium-enhanced MRI has largely replaced myelography for this purpose, and CSF cytology is obtained postoperatively as a routine procedure.

In other embodiments, the subject method is used as part of treatment program for ependymomas. Ependymomas account for approximately 10% of the

pediatric brain tumors in children. Grossly, they are tumors that arise from the ependymal lining of the ventricles and microscopically form rosettes, canals, and perivascular rosettes. In the CHOP series of 51 children reported with ependymomas,  $\frac{3}{4}$  were histologically benign. Approximately 2/3 arose from the 5 region of the 4th ventricle. One third presented in the supratentorial region. Age at presentation peaks between birth and 4 years, as demonstrated by SEER data as well as data from CHOP. The median age is about 5 years. Because so many children with this disease are babies, they often require multimodal therapy.

Yet another aspect of the present invention concerns the observation in the 10 art that *ptc*, *hedgehog*, and/or *smoothened* are involved in morphogenic signals involved in other vertebrate organogenic pathways in addition to neuronal differentiation as described above, having apparent roles in other endodermal patterning, as well as both mesodermal and endodermal differentiation processes. Thus, it is contemplated by the invention that compositions comprising *hedgehog* 15 antagonists can also be utilized for both cell culture and therapeutic methods involving generation and maintenance of non-neuronal tissue.

In one embodiment, the present invention makes use of the discovery that *ptc*, *hedgehog*, and *smoothened* are apparently involved in controlling the development of stem cells responsible for formation of the digestive tract, liver, 20 lungs, and other organs which derive from the primitive gut. *Shh* serves as an inductive signal from the endoderm to the mesoderm, which is critical to gut morphogenesis. Therefore, for example, *hedgehog* antagonists of the instant method can be employed for regulating the development and maintenance of an artificial liver that can have multiple metabolic functions of a normal liver. In an exemplary 25 embodiment, the subject method can be used to regulate the proliferation and differentiation of digestive tube stem cells to form hepatocyte cultures which can be used to populate extracellular matrices, or which can be encapsulated in biocompatible polymers, to form both implantable and extracorporeal artificial livers.

In another embodiment, therapeutic compositions of *hedgehog* antagonists can be utilized in conjunction with transplantation of such artificial livers, as well as embryonic liver structures, to regulate uptake of intraperitoneal implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted liver  
5 tissue.

In yet another embodiment, the subject method can be employed therapeutically to regulate such organs after physical, chemical or pathological insult. For instance, therapeutic compositions comprising *hedgehog* antagonists can be utilized in liver repair subsequent to a partial hepatectomy.

10 The generation of the pancreas and small intestine from the embryonic gut depends on intercellular signalling between the endodermal and mesodermal cells of the gut. In particular, the differentiation of intestinal mesoderm into smooth muscle has been suggested to depend on signals from adjacent endodermal cells. One candidate mediator of endodermally derived signals in the embryonic hindgut is  
15 Sonic hedgehog. See, for example, Apelqvist et al. (1997) *Curr Biol* 7:801-4. The Shh gene is expressed throughout the embryonic gut endoderm with the exception of the pancreatic bud endoderm, which instead expresses high levels of the homeodomain protein *Ipfl/Pdx1* (insulin promoter factor 1/pancreatic and duodenal homeobox 1), an essential regulator of early pancreatic development. Apelqvist et  
20 al., *supra*, have examined whether the differential expression of Shh in the embryonic gut tube controls the differentiation of the surrounding mesoderm into specialised mesoderm derivatives of the small intestine and pancreas. To test this, they used the promoter of the *Ipfl/Pdx1* gene to selectively express Shh in the developing pancreatic epithelium. In *Ipfl/Pdx1*- Shh transgenic mice, the pancreatic  
25 mesoderm developed into smooth muscle and interstitial cells of Cajal, characteristic of the intestine, rather than into pancreatic mesenchyme and spleen. Also, pancreatic explants exposed to Shh underwent a similar program of intestinal differentiation. These results provide evidence that the differential expression of endodermally derived Shh controls the fate of adjacent mesoderm at different regions of the gut  
30 tube.

In the context of the present invention, it is contemplated therefore that the subject *hedgehog* antagonists can be used to control or regulate the proliferation and/or differentiation of pancreatic tissue both *in vivo* and *in vitro*.

There are a wide variety of pathological cell proliferative and differentiative 5 conditions for which the inhibitors of the present invention may provide therapeutic benefits, with the general strategy being, for example, the correction of aberrant insulin expression, or modulation of differentiation. More generally, however, the present invention relates to a method of inducing and/or maintaining a differentiated state, enhancing survival and/or affecting proliferation of pancreatic cells, by 10 contacting the cells with the subject inhibitors. For instance, it is contemplated by the invention that, in light of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in the formation of ordered spatial arrangements of pancreatic tissues, the subject method could be used as part of a technique to generate and/or maintain such tissue both *in vitro* and *in vivo*. For instance, modulation of the function of 15 *hedgehog* can be employed in both cell culture and therapeutic methods involving generation and maintenance  $\beta$ -cells and possibly also for non-pancreatic tissue, such as in controlling the development and maintenance of tissue from the digestive tract, spleen, lungs, urogenital organs (e.g., bladder), and other organs which derive from the primitive gut.

20 In an exemplary embodiment, the present method can be used in the treatment of hyperplastic and neoplastic disorders effecting pancreatic tissue, particularly those characterized by aberrant proliferation of pancreatic cells. For instance, pancreatic cancers are marked by abnormal proliferation of pancreatic cells that can result in alterations of insulin secretory capacity of the pancreas. For 25 instance, certain pancreatic hyperplasias, such as pancreatic carcinomas, can result in hypoinsulinemia due to dysfunction of  $\beta$ -cells or decreased islet cell mass. To the extent that aberrant *ptc*, *hedgehog*, and *smoothened* signaling may be indicated in disease progression, the subject inhibitors, can be used to enhance regeneration of the tissue after anti-tumor therapy.

Moreover, manipulation of *hedgehog* signaling properties at different points may be useful as part of a strategy for reshaping/repairing pancreatic tissue both *in vivo* and *in vitro*. In one embodiment, the present invention makes use of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in regulating the 5 development of pancreatic tissue. In general, the subject method can be employed therapeutically to regulate the pancreas after physical, chemical or pathological insult. In yet another embodiment, the subject method can be applied to cell culture techniques, and in particular, may be employed to enhance the initial 10 generation of prosthetic pancreatic tissue devices. Manipulation of proliferation and differentiation of pancreatic tissue, for example, by altering *hedgehog* activity, can provide a means for more carefully controlling the characteristics of a cultured tissue. In an exemplary embodiment, the subject method can be used to augment 15 production of prosthetic devices which require  $\beta$ -islet cells, such as may be used in the encapsulation devices described in, for example, the Aebischer et al. U.S. Patent No. 4,892,538, the Aebischer et al. U.S. Patent No. 5,106,627, the Lim U.S. Patent No. 4,391,909, and the Sefton U.S. Patent No. 4,353,888. Early progenitor cells to the pancreatic islets are multipotential, and apparently coactivate all the islet-specific genes from the time they first appear. As development proceeds, expression of islet-specific hormones, such as insulin, becomes restricted to the pattern of expression 20 characteristic of mature islet cells. The phenotype of mature islet cells, however, is not stable in culture, as reappearance of embryonal traits in mature  $\beta$ -cells can be observed. By utilizing the subject hedgehog antagonists, the differentiation path or proliferative index of the cells can be regulated.

Furthermore, manipulation of the differentiative state of pancreatic tissue can 25 be utilized in conjunction with transplantation of artificial pancreas so as to promote implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted tissue. For instance, manipulation of *hedgehog* function to affect tissue differentiation can be utilized as a means of maintaining graft viability.

Bellusci et al. (1997) *Development* 124:53 report that *Sonic hedgehog* 30 regulates lung mesenchymal cell proliferation *in vivo*. Accordingly, the present

method can be used to regulate regeneration of lung tissue, e.g., in the treatment of emphysema and respiratory distress syndrome.

Fujita et al. (1997) *Biochem Biophys Res Commun* 238:658 reported that Sonic hedgehog is expressed in human lung squamous carcinoma and 5 adenocarcinoma cells. The expression of Sonic hedgehog was also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. They also observed that Sonic hedgehog stimulates the incorporation of BrdU into the carcinoma cells and stimulates their cell growth, while anti-Shh-N inhibited their cell growth. These results suggest that a *ptc*, *hedgehog*, and/or 10 *smoothened* is involved in the cell growth of such transformed lung tissue and therefore indicates that the subject method can be used as part of a treatment of lung carcinoma and adenocarcinomas, and other proliferative disorders involving the lung epithelia.

Many other tumors may, based on evidence such as involvement of the 15 hedgehog pathway in these tumors, or detected expression of hedgehog or its receptor in these tissues during development, be affected by treatment with the subject compounds. Such tumors include, but are by no means limited to, tumors related to Gorlin's syndrome (e.g., basal cell carcinoma, medulloblastoma, meningioma, etc.), tumors evidenced in *ptc* knock-out mice (e.g., hemangioma, 20 rhabdomyosarcoma, etc.), tumors resulting from *gli-1* amplification (e.g., glioblastoma, sarcoma, etc.), tumors connected with TRC8, a *ptc* homolog (e.g., renal carcinoma, thyroid carcinoma, etc.), *Ext-1*-related tumors (e.g., bone cancer, etc.), Shh-induced tumors (e.g., lung cancer, chondrosarcomas, etc.), and other tumors (e.g., breast cancer, urogenital cancer (e.g., kidney, bladder, ureter, prostate, 25 etc.), fibrosarcoma, hypothalamic hamartoma, adrenal cancer, gastrointestinal cancer (e.g., stomach, intestine (including parts of the small and large intestine), esophagus, and gut annexes such as pancreas, the biliary duct system, etc.), etc.).

In still another embodiment of the present invention, compositions comprising *hedgehog* antagonists can be used in the *in vitro* generation of skeletal 30 tissue, such as from skeletogenic stem cells, as well as the *in vivo* treatment of

skeletal tissue deficiencies. The present invention particularly contemplates the use of *hedgehog* antagonists to regulate the rate of chondrogenesis and/or osteogenesis. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective 5 tissue, no matter how the deficiency originated, e.g. whether as a result of surgical intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions.

For instance, the method of the present invention can be used as part of a regimen for restoring cartilage function to a connective tissue. Such methods are 10 useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a laxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. The present 15 reparative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as periodontal surgery. The present method may also be applied to improving a previous reparative procedure, for example, following surgical repair of a meniscus, ligament, or cartilage. Furthermore, it may prevent the onset or exacerbation of degenerative disease if applied early enough after trauma.

20 In one embodiment of the present invention, the subject method comprises treating the afflicted connective tissue with a therapeutically sufficient amount of a *hedgehog* antagonist, particularly an antagonist selective for Indian *hedgehog* signal transduction, to regulate a cartilage repair response in the connective tissue by managing the rate of differentiation and/or proliferation of chondrocytes embedded 25 in the tissue. Such connective tissues as articular cartilage, interarticular cartilage (menisci), costal cartilage (connecting the true ribs and the sternum), ligaments, and tendons are particularly amenable to treatment in reconstructive and/or regenerative therapies using the subject method. As used herein, regenerative therapies include treatment of degenerative states which have progressed to the point of which

impairment of the tissue is obviously manifest, as well as preventive treatments of tissue where degeneration is in its earliest stages or imminent.

In an illustrative embodiment, the subject method can be used as part of a therapeutic intervention in the treatment of cartilage of a diarthroidal joint, such as a knee, an ankle, an elbow, a hip, a wrist, a knuckle of either a finger or toe, or a tempomandibular joint. The treatment can be directed to the meniscus of the joint, to the articular cartilage of the joint, or both. To further illustrate, the subject method can be used to treat a degenerative disorder of a knee, such as which might be the result of traumatic injury (e.g., a sports injury or excessive wear) or osteoarthritis.

10 The subject antagonists may be administered as an injection into the joint with, for instance, an arthroscopic needle. In some instances, the injected agent can be in the form of a hydrogel or other slow release vehicle described above in order to permit a more extended and regular contact of the agent with the treated tissue.

The present invention further contemplates the use of the subject method in the field of cartilage transplantation and prosthetic device therapies. However, problems arise, for instance, because the characteristics of cartilage and fibrocartilage varies between different tissue: such as between articular, meniscal cartilage, ligaments, and tendons, between the two ends of the same ligament or tendon, and between the superficial and deep parts of the tissue. The zonal arrangement of these tissues may reflect a gradual change in mechanical properties, and failure occurs when implanted tissue, which has not differentiated under those conditions, lacks the ability to appropriately respond. For instance, when meniscal cartilage is used to repair anterior cruciate ligaments, the tissue undergoes a metaplasia to pure fibrous tissue. By regulating the rate of chondrogenesis, the subject method can be used to particularly address this problem, by helping to adaptively control the implanted cells in the new environment and effectively resemble hypertrophic chondrocytes of an earlier developmental stage of the tissue.

In similar fashion, the subject method can be applied to enhancing both the generation of prosthetic cartilage devices and to their implantation. The need for improved treatment has motivated research aimed at creating new cartilage that is

based on collagen-glycosaminoglycan templates (Stone et al. (1990) *Clin Orthop Relat Res* 252:129), isolated chondrocytes (Grande et al. (1989) *J Orthop Res* 7:208; and Takigawa et al. (1987) *Bone Miner* 2:449), and chondrocytes attached to natural or synthetic polymers (Walitani et al. (1989) *J Bone Jt Surg* 71B:74; Vacanti et al. 5 (1991) *Plast Reconstr Surg* 88:753; von Schroeder et al. (1991) *J Biomed Mater Res* 25:329; Freed et al. (1993) *J Biomed Mater Res* 27:11; and the Vacanti et al. U.S. Patent No. 5,041,138). For example, chondrocytes can be grown in culture on biodegradable, biocompatible highly porous scaffolds formed from polymers such as polyglycolic acid, polylactic acid, agarose gel, or other polymers that degrade over 10 time as function of hydrolysis of the polymer backbone into innocuous monomers. The matrices are designed to allow adequate nutrient and gas exchange to the cells until engraftment occurs. The cells can be cultured *in vitro* until adequate cell volume and density has developed for the cells to be implanted. One advantage of the matrices is that they can be cast or molded into a desired shape on an individual 15 basis, so that the final product closely resembles the patient's own ear or nose (by way of example), or flexible matrices can be used which allow for manipulation at the time of implantation, as in a joint.

In one embodiment of the subject method, the implants are contacted with a *hedgehog* antagonist during certain stages of the culturing process in order to 20 manage the rate of differentiation of chondrocytes and the formation of hypertrophic chondrocytes in the culture.

In another embodiment, the implanted device is treated with a *hedgehog* antagonist in order to actively remodel the implanted matrix and to make it more suitable for its intended function. As set out above with respect to tissue transplants, 25 the artificial transplants suffer from the same deficiency of not being derived in a setting which is comparable to the actual mechanical environment in which the matrix is implanted. The ability to regulate the chondrocytes in the matrix by the subject method can allow the implant to acquire characteristics similar to the tissue for which it is intended to replace.

In yet another embodiment, the subject method is used to enhance attachment of prosthetic devices. To illustrate, the subject method can be used in the implantation of a periodontal prosthesis, wherein the treatment of the surrounding connective tissue stimulates formation of periodontal ligament about the prosthesis.

5        In still further embodiments, the subject method can be employed as part of a regimen for the generation of bone (osteogenesis) at a site in the animal where such skeletal tissue is deficient. Indian hedgehog is particularly associated with the hypertrophic chondrocytes that are ultimately replaced by osteoblasts. For instance, administration of a *hedgehog* antagonist of the present invention can be employed as  
10      10 part of a method for regulating the rate of bone loss in a subject. For example, preparations comprising *hedgehog* antagonists can be employed, for example, to control endochondral ossification in the formation of a "model" for ossification.

In yet another embodiment of the present invention, a *hedgehog* antagonist can be used to regulate spermatogenesis. The *hedgehog* proteins, particularly Dhh, 15      15 have been shown to be involved in the differentiation and/or proliferation and maintenance of testicular germ cells. Dhh expression is initiated in Sertoli cell precursors shortly after the activation of Sry (testicular determining gene) and persists in the testis into the adult. Males are viable but infertile, owing to a complete absence of mature sperm. Examination of the developing testis in different genetic 20      20 backgrounds suggests that Dhh regulates both early and late stages of spermatogenesis. Bitgood et al. (1996) *Curr Biol* 6:298. In a preferred embodiment, the *hedgehog* antagonist can be used as a contraceptive. In similar fashion, *hedgehog* antagonists of the subject method are potentially useful for modulating normal ovarian function.

25        The subject method also has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of administering to an animal an amount of a *hedgehog* antagonist effective to alter the growth state of a treated epithelial tissue. The mode of administration and dosage regimens will vary  
30      30 depending on the epithelial tissue(s) to be treated. For example, topical formulations

will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method that "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the  
5 absence of the treatment. "Promotion of wound healing" can also mean that the method regulates the proliferation and/or growth of, *inter alia*, keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved  
10 success rates, (e.g., the take rates of skin grafts,) when used together with the method of the present invention.

Despite significant progress in reconstructive surgical techniques, scarring can be an important obstacle in regaining normal function and appearance of healed skin. This is particularly true when pathologic scarring such as keloids or  
15 hypertrophic scars of the hands or face causes functional disability or physical deformity. In the severest circumstances, such scarring may precipitate psychosocial distress and a life of economic deprivation. Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. Through  
20 the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.

The present treatment can also be effective as part of a therapeutic regimen for treating oral and paraoral ulcers, e.g. resulting from radiation and/or  
25 chemotherapy. Such ulcers commonly develop within days after chemotherapy or radiation therapy. These ulcers usually begin as small, painful irregularly shaped lesions usually covered by a delicate gray necrotic membrane and surrounded by inflammatory tissue. In many instances, lack of treatment results in proliferation of tissue around the periphery of the lesion on an inflammatory basis. For instance, the  
30 epithelium bordering the ulcer usually demonstrates proliferative activity, resulting

in loss of continuity of surface epithelium. These lesions, because of their size and loss of epithelial integrity, dispose the body to potential secondary infection. Routine ingestion of food and water becomes a very painful event and, if the ulcers proliferate throughout the alimentary canal, diarrhea usually is evident with all its 5 complicating factors. According to the present invention, a treatment for such ulcers which includes application of a *hedgehog* antagonist can reduce the abnormal proliferation and differentiation of the affected epithelium, helping to reduce the severity of subsequent inflammatory events.

The subject method and compositions can also be used to treat wounds 10 resulting from dermatological diseases, such as lesions resulting from autoimmune disorders such as psoriasis. Atopic dermatitis refers to skin trauma resulting from allergies associated with an immune response caused by allergens such as pollens, foods, dander, insect venoms and plant toxins.

In other embodiments, antiproliferative preparations of *hedgehog* antagonists 15 can be used to inhibit lens epithelial cell proliferation to prevent post-operative complications of extracapsular cataract extraction. Cataract is an intractable eye disease and various studies on a treatment of cataract have been made. But at present, the treatment of cataract is attained by surgical operations. Cataract surgery has been applied for a long time and various operative methods have been examined. 20 Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidence of aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber type intraocular lenses that are now considered to be the lenses of choice in 25 most cases.

However, a disadvantage of extracapsular cataract extraction is the high 30 incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within three years after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells that remain after extracapsular lens extraction. These cells proliferate to cause

Sommerling rings, and along with fibroblasts that also deposit and occur on the posterior capsule, cause opacification of the posterior capsule, which interferes with vision. Prevention of after-cataract would be preferable to treatment. To inhibit secondary cataract formation, the subject method provides a means for inhibiting 5 proliferation of the remaining lens epithelial cells. For example, such cells can be induced to remain quiescent by instilling a solution containing an *hedgehog* antagonist preparation into the anterior chamber of the eye after lens removal. Furthermore, the solution can be osmotically balanced to provide minimal effective dosage when instilled into the anterior chamber of the eye, thereby inhibiting 10 subcapsular epithelial growth with some specificity.

The subject method can also be used in the treatment of corneopathies marked by corneal epithelial cell proliferation, as for example in ocular epithelial disorders such as epithelial downgrowth or squamous cell carcinomas of the ocular surface.

15 Levine et al. (1997) *J Neurosci* 17:6277 show that hedgehog proteins can regulate mitogenesis and photoreceptor differentiation in the vertebrate retina, and Ihh is a candidate factor from the pigmented epithelium to promote retinal progenitor proliferation and photoreceptor differentiation. Likewise, Jensen et al. (1997) *Development* 124:363 demonstrated that treatment of cultures of perinatal mouse 20 retinal cells with the amino-terminal fragment of Sonic hedgehog protein results in an increase in the proportion of cells that incorporate bromodeoxyuridine, in total cell numbers, and in rod photoreceptors, amacrine cells and Muller glial cells, suggesting that Sonic hedgehog promotes the proliferation of retinal precursor cells. Thus, the subject method can be used in the treatment of proliferative diseases of retinal cells 25 and regulate photoreceptor differentiation.

Yet another aspect of the present invention relates to the use of the subject method to control hair growth. Hair is basically composed of keratin, a tough and insoluble protein; its chief strength lies in its disulphide bond of cystine. Each individual hair comprises a cylindrical shaft and a root, and is contained in a follicle, 30 a flask-like depression in the skin. The bottom of the follicle contains a finger-like

projection termed the papilla, which consists of connective tissue from which hair grows, and through which blood vessels supply the cells with nourishment. The shaft is the part that extends outwards from the skin surface, whilst the root has been described as the buried part of the hair. The base of the root expands into the hair 5 bulb, which rests upon the papilla. Cells from which the hair is produced grow in the bulb of the follicle; they are extruded in the form of fibers as the cells proliferate in the follicle. Hair "growth" refers to the formation and elongation of the hair fiber by the dividing cells.

As is well known in the art, the common hair cycle is divided into three 10 stages: anagen, catagen and telogen. During the active phase (anagen), the epidermal stem cells of the dermal papilla divide rapidly. Daughter cells move upward and differentiate to form the concentric layers of the hair itself. The transitional stage, catagen, is marked by the cessation of mitosis of the stem cells in the follicle. The resting stage is known as telogen, where the hair is retained within the scalp for 15 several weeks before an emerging new hair developing below it dislodges the telogen-phase shaft from its follicle. From this model it has become clear that the larger the pool of dividing stem cells that differentiate into hair cells, the more hair growth occurs. Accordingly, methods for increasing or reducing hair growth can be carried out by potentiating or inhibiting, respectively, the proliferation of these stem 20 cells.

In certain embodiments, the subject method can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g. 25 hypertrichosis. In an exemplary embodiment, *hedgehog* antagonists can be used to manage hirsutism, a disorder marked by abnormal hairiness. The subject method can also provide a process for extending the duration of depilation.

Moreover, because a *hedgehog* antagonist will often be cytostatic to epithelial cells, rather than cytotoxic, such agents can be used to protect hair follicle 30 cells from cytotoxic agents that require progression into S-phase of the cell-cycle for

efficacy, e.g. radiation-induced death. Treatment by the subject method can provide protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, *hedgehog* antagonists can be used for patients undergoing chemo- or radiation-therapies that ordinarily result in hair loss. By inhibiting cell-cycle progression during such therapies, the subject treatment can protect hair follicle cells from death that might otherwise result from activation of cell death programs. After the therapy has concluded, the instant method can also be removed with concomitant relief of the inhibition of follicle cell proliferation.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematosa reticulata or keloid folliculitis. For example, a cosmetic preparation of an *hedgehog* antagonist can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In another aspect of the invention, the subject method can be used to induce differentiation and/or inhibit proliferation of epithelially derived tissue. Such forms of these molecules can provide a basis for differentiation therapy for the treatment of hyperplastic and/or neoplastic conditions involving epithelial tissue. For example, such preparations can be used for the treatment of cutaneous diseases in which there is abnormal proliferation or growth of cells of the skin.

For instance, the pharmaceutical preparations of the invention are intended for the treatment of hyperplastic epidermal conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, as for example basal cell carcinoma or squamous cell carcinoma. The subject method can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis.

Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthoma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate 5 much more rapidly than normal and to differentiate less completely.

In one embodiment, the preparations of the present invention are suitable for the treatment of dermatological ailments linked to keratinization disorders causing abnormal proliferation of skin cells, which disorders may be marked by either inflammatory or non-inflammatory components. To illustrate, therapeutic 10 preparations of a *hedgehog* antagonist, e.g., which promotes quiescence or differentiation can be used to treat varying forms of psoriasis, be they cutaneous, mucosal or ungual. Psoriasis, as described above, is typically characterized by epidermal keratinocytes that display marked proliferative activation and differentiation along a "regenerative" pathway. Treatment with an antiproliferative 15 embodiment of the subject method can be used to reverse the pathological epidermal activation and can provide a basis for sustained remission of the disease.

A variety of other keratotic lesions are also candidates for treatment with the subject method. Actinic keratoses, for example, are superficial inflammatory premalignant tumors arising on sun-exposed and irradiated skin. The lesions are 20 erythematous to brown with variable scaling. Current therapies include excisional and cryosurgery. These treatments are painful, however, and often produce cosmetically unacceptable scarring. Accordingly, treatment of keratosis, such as actinic keratosis, can include application, preferably topical, of a *hedgehog* antagonist composition in amounts sufficient to inhibit hyperproliferation of 25 epidermal/epidermoid cells of the lesion.

Acne represents yet another dermatologic ailment which may be treated by the subject method. Acne vulgaris, for instance, is a multifactorial disease most commonly occurring in teenagers and young adults, and is characterized by the appearance of inflammatory and noninflammatory lesions on the face and upper 30 trunk. The basic defect which gives rise to acne vulgaris is hypercornification of the duct of a hyperactive sebaceous gland. Hypercornification blocks the normal

mobility of skin and follicle microorganisms, and in so doing, stimulates the release of lipases by *Propinobacterium acnes* and *Staphylococcus epidermidis* bacteria and *Pitrosporum ovale*, a yeast. Treatment with an antiproliferative *hedgehog* antagonist, particularly topical preparations, may be useful for preventing the transitional 5 features of the ducts, e.g. hypercornification, which lead to lesion formation. The subject treatment may further include, for example, antibiotics, retinoids and antiandrogens.

The present invention also provides a method for treating various forms of dermatitis. Dermatitis is a descriptive term referring to poorly demarcated lesions 10 that are either pruritic, erythematous, scaly, blistered, weeping, fissured or crusted. These lesions arise from any of a wide variety of causes. The most common types of dermatitis are atopic, contact and diaper dermatitis. For instance, seborrheic dermatitis is a chronic, usually pruritic, dermatitis with erythema, dry, moist, or greasy scaling, and yellow crusted patches on various areas, especially the scalp, 15 with exfoliation of an excessive amount of dry scales. The subject method can also be used in the treatment of stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves or x- or gamma-radiation. According to the present invention, the subject method can be used in the treatment and/or 20 prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipuritics, and antibiotics.

For example, it is contemplated that the subject method could be used to inhibit angiogenesis. Hedgehog is known to stimulate angiogenesis. Matrigel plugs 25 impregnated with hedgehog protein and inserted into mice evince substantial neovascularization, whereas Matrigel plugs not carrying hedgehog show comparatively little vascularization. Hedgehog protein is also capable of increasing vascularization of the normally avascular mouse cornea. The *ptc-1* gene is expressed in normal vascular tissues, including the endothelial cells of the aorta, vascular 30 smooth muscle cells, adventitial fibroblasts of the aorta, the coronary vasculature and cardiomyocytes of the atria and ventricles. These tissues are also sensitive to

hedgehog protein. Treatment with exogenous hedgehog causes upregulation of *ptc-1* expression. In addition, hedgehog proteins stimulate proliferation of vascular smooth muscle cells in vivo. Hedgehog proteins also cause fibroblasts to increase expression of angiogenic growth factors such as VEGF, bFGF, Ang-1 and Ang-2. Lastly, 5 hedgehog proteins are known to stimulate recovery from ischemic injury and stimulate formation of collateral vessels.

Given that hedgehog promotes angiogenesis, hedgehog antagonists are expected to act as angiogenesis inhibitors, particularly in situations where some level of hedgehog signaling is necessary for angiogenesis.

10 Angiogenesis is fundamental to many disorders. Persistent, unregulated angiogenesis occurs in a range of disease states, tumor metastases and abnormal growths by endothelial cells. The vasculature created as a result of angiogenic processes supports the pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped 15 together as angiogenic dependent or angiogenic associated diseases. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Diseases caused by, supported by or associated with angiogenesis include 20 ocular neovascular disease, age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrobulbar fibroplasia, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, Sjogren's, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex 25 infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Stevens Johnson disease, periphigoid radial keratotomy, corneal graft rejection, rheumatoid arthritis, osteoarthritis chronic inflammation (eg., ulcerative colitis or Crohn's

disease), hemangioma, Osler-Weber-Rendu disease, and hereditary hemorrhagic telangiectasia.

In addition, angiogenesis plays a critical role in cancer. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes.

5    Tumors in which angiogenesis is important include solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas. Angiogenic factors have been found associated with several solid tumors. Prevention of angiogenesis could halt the growth of these

10    tumors and the resultant damage to the animal due to the presence of the tumor. Angiogenesis is also associated with blood-born tumors such as leukemias, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and

15    spleen. It is believed that angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia-like tumors.

In addition to tumor growth, angiogenesis is important in metastasis. Initially, angiogenesis is important in the vascularization of the tumor which allows cancerous cells to enter the blood stream and to circulate throughout the

20    body. After the tumor cells have left the primary site, and have settled into the secondary, metastasis site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis could lead to the prevention of metastasis of tumors and possibly contain the neoplastic growth at the primary site.

Angiogenesis is also involved in normal physiological processes such as

25    reproduction and wound healing. Angiogenesis is an important step in ovulation and also in implantation of the blastula after fertilization. Prevention of angiogenesis could be used to induce amenorrhea, to block ovulation or to prevent implantation by the blastula.

It is anticipated that the invention will be useful for the treatment and/or

30    prevention of respiratory distress syndrome or other disorders resulting from

inappropriate lung surface tension. Respiratory distress syndrome results from insufficient surfactant in the alveolae of the lungs. The lungs of vertebrates contain surfactant, a complex mixture of lipids and protein that causes surface tension to rise during lung inflation and decrease during lung deflation. During lung deflation, 5 surfactant decreases such that there are no surface forces that would otherwise promote alveolar collapse. Aerated alveoli that have not collapsed during expiration permit continuous oxygen and carbon dioxide transport between blood and alveolar gas and require much less force to inflate during the subsequent inspiration. During inflation, lung surfactant increases surface tension as the alveolar surface area 10 increases. A rising surface tension in expanding alveoli opposes over-inflation in those airspaces and tends to divert inspired air to less well-aerated alveoli, thereby facilitating even lung aeration.

Respiratory distress syndrome is particularly prevalent among premature infants. Lung surfactant is normally synthesized at a very low rate until the last six 15 weeks of fetal life. Human infants born more than six weeks before the normal term of a pregnancy have a high risk of being born with inadequate amounts of lung surfactant and inadequate rates of surfactant synthesis. The more prematurely an infant is born, the more severe the surfactant deficiency is likely to be. Severe surfactant deficiency can lead to respiratory failure within a few minutes or hours of 20 birth. The surfactant deficiency produces progressive collapse of alveoli (atelectasis) because of the decreasing ability of the lung to expand despite maximum inspiratory effort. As a result, inadequate amounts of oxygen reach the infant's blood. RDS can occur in adults as well, typically as a consequence of failure in surfactant biosynthesis.

25 Lung tissue of premature infants shows high activity of the hedgehog signaling pathway. Inhibition of this pathway using hedgehog antagonists increases the formation of lamellar bodies and increases the expression of genes involved in surfactant biosynthesis. Lamellar bodies are subcellular structures associated with surfactant biosynthesis. For these reasons, treatment of premature infants with a 30 hedgehog antagonist should stimulate surfactant biosynthesis and ameliorate RDS.

In cases where adult RDS is associated with hedgehog pathway activation, treatment with hedgehog antagonists should also be effective.

It is further contemplated that the use of hedgehog antagonists may be specifically targeted to disorders where the affected tissue and/or cells evince high hedgehog pathway activation. Expression of *gli* genes is activated by the hedgehog signaling pathway, including *gli-1*, *gli-2* and *gli-3*. *gli-1* expression is most consistently correlated with hedgehog signaling activity across a wide range of tissues and disorders, while *gli-3* is somewhat less so. The *gli* genes encode transcription factors that activate expression of many genes needed to elicit the full effects of hedgehog signaling. However, the Gli-3 transcription factor can also act as a repressor of hedgehog effector genes, and therefore, expression of *gli-3* can cause a decreased effect of the hedgehog signaling pathway. Whether Gli-3 acts as a transcriptional activator or repressor depends on post-translational events, and therefore it is expected that methods for detecting the activating form (versus the repressing form) of Gli-3 protein would also be a reliable measure of hedgehog pathway activation. *gli-2* gene expression is expected to provide a reliable marker for hedgehog pathway activation. The *gli-1* gene is strongly expressed in a wide array of cancers, hyperplasias and immature lungs, and serves as a marker for the relative activation of the hedgehog pathway. In addition, tissues, such as immature lung, that have high *gli* gene expression are strongly affected by hedgehog inhibitors. Accordingly, it is contemplated that the detection of *gli* gene expression may be used as a powerful predictive tool to identify tissues and disorders that will particularly benefit from treatment with a hedgehog antagonist.

In preferred embodiments, *gli-1* expression levels are detected, either by direct detection of the transcript or by detection of protein levels or activity. Transcripts may be detected using any of a wide range of techniques that depend primarily on hybridization of probes to the *gli-1* transcripts or to cDNAs synthesized therefrom. Well known techniques include Northern blotting, reverse-transcriptase PCR and microarray analysis of transcript levels. Methods for detecting Gli protein levels include Western blotting, immunoprecipitation, two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)(preferably compared against a

standard wherein the position of the Gli proteins has been determined), and mass spectroscopy. Mass spectroscopy may be coupled with a series of purification steps to allow high-throughput identification of many different protein levels in a particular sample. Mass spectroscopy and 2D SDS-PAGE can also be used to

5 identify post-transcriptional modifications to proteins including proteolytic events, ubiquitination, phosphorylation, lipid modification etc. Gli activity may also be assessed by analyzing binding to substrate DNA or in vitro transcriptional activation of target promoters. Gel shift assays, DNA footprinting assays and DNA-protein crosslinking assays are all methods that may be used to assess the presence of a

10 protein capable of binding to Gli binding sites on DNA.

In preferred embodiments, *gli* transcript levels are measured and diseased or disordered tissues showing abnormally high *gli* levels are treated with a hedgehog antagonist. Premature lung tissue, lung cancers (e.g., adenocarcinomas, broncho-alveolar adenocarcinomas, small cell carcinomas), breast cancers (e.g., inferior ductal carcinomas, inferior lobular carcinomas, tubular carcinomas), prostate cancers (e.g., adenocarcinomas), and benign prostatic hyperplasias all show strongly elevated *gli-1* expression levels in certain cases. Accordingly, *gli-1* expression levels are a powerful diagnostic device to determine which of these tissues should be treated with a hedgehog antagonist. In addition, there is substantial correlative evidence that

15 cancers of urothelial cells (e.g., bladder cancer, other urogenital cancers) will also have elevated *gli-1* levels in certain cases. For example, it is known that loss of heterozygosity on chromosome 9q22 is common in bladder cancers. The *ptc-1* gene is located at this position and *ptc-1* loss of function is probably a partial cause of hyperproliferation, as in many other cancer types. Accordingly, such cancers would

20 also show high *gli* expression and would be particularly amenable to treatment with a hedgehog antagonist.

25

Expression of *ptc-1* and *ptc-2* is also activated by the hedgehog signaling pathway, but these genes are inferior to the *gli* genes as markers of hedgehog pathway activation. In certain tissues only one of *ptc-1* or *ptc-2* is expressed

30 although the hedgehog pathway is highly active. For example, in testicular development, Indian hedgehog plays an important role and the hedgehog pathway is

activated, but only *ptc-2* is expressed. Accordingly, these genes are individually unreliable as markers for hedgehog pathway activation, although simultaneous measurement of both genes are contemplated as a useful indicator for tissues to be treated with a hedgehog antagonist.

5 Ailments which may be treated by the subject method are disorders specific to non-humans, such as mange.

In still another embodiment, the subject method can be used in the treatment of human cancers, particularly basal cell carcinomas and other tumors of epithelial tissues such as the skin. For example, *hedgehog* antagonists can be employed, in the 10 subject method, as part of a treatment for basal cell nevus syndrome (BCNS), and other other human carcinomas, adenocarcinomas, sarcomas and the like.

In a preferred embodiment, the subject method is used as part of a treatment or prophylaxis regimen for treating (or preventing) basal cell carcinoma. The 15 deregulation of the *hedgehog* signaling pathway may be a general feature of basal cell carcinomas caused by *ptc* mutations. Consistent overexpression of human *ptc* mRNA has been described in tumors of familial and sporadic BCCs, determined by in situ hybridization. Mutations that inactivate *ptc* may be expected to result in overexpression of mutant *Ptc*, because *ptc* displays negative autoregulation. Prior research demonstrates that overexpression of hedgehog proteins can also lead to 20 tumorigenesis. That sonic hedgehog (*Shh*) has a role in tumorigenesis in the mouse has been suggested by research in which transgenic mice overexpressing *Shh* in the skin developed features of BCNS, including multiple BCC-like epidermal proliferations over the entire skin surface, after only a few days of skin development. A mutation in the *Shh* human gene from a BCC was also described; it was suggested 25 that *Shh* or other *Hh* genes in humans could act as dominant oncogenes in humans. Sporadic *ptc* mutations have also been observed in BCCs from otherwise normal individuals, some of which are UV-signature mutations. In one recent study of sporadic BCCs, five UV-signature type mutations, either CT or CCTT changes, were found out of fifteen tumors determined to contain *ptc* mutations. Another recent 30 analysis of sporadic *ptc* mutations in BCCs and neuroectodermal tumors revealed

one CT change in one of three *ptc* mutations found in the BCCs. See, for example, Goodrich et al. (1997) *Science* 277:1109-13; Xie et al. (1997) *Cancer Res* 57:2369-72; Oro et al. (1997) *Science* 276:817-21; Xie et al. (1997) *Genes Chromosomes Cancer* 18:305-9; Stone et al. (1996) *Nature* 384:129-34; and Johnson et al. (1996) 5 *Science* 272:1668-71.

The subject method can also be used to treat patients with BCNS, e.g., to prevent BCC or other effects of the disease which may be the result of *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function. Basal cell nevus syndrome is a rare autosomal dominant disorder characterized by multiple 10 BCCs that appear at a young age. BCNS patients are very susceptible to the development of these tumors; in the second decade of life, large numbers appear, mainly on sun-exposed areas of the skin. This disease also causes a number of developmental abnormalities, including rib, head and face alterations, and sometimes polydactyly, syndactyly, and spina bifida. They also develop a number of 15 tumor types in addition to BCCs: fibromas of the ovaries and heart, cysts of the skin and jaws, and in the central nervous system, medulloblastomas and meningiomas. The subject method can be used to prevent or treat such tumor types in BCNS and non-BCNS patients. Studies of BCNS patients show that they have both genomic and sporadic mutations in the *ptc* gene, suggesting that these mutations are the 20 ultimate cause of this disease.

In another aspect, the present invention provides pharmaceutical preparations comprising *hedgehog* antagonists. The *hedgehog* antagonists for use in the subject method may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, 25 propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all 30 solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any

conventional media or agent is incompatible with the activity of the *hedgehog* antagonist, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* 5 (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations".

Pharmaceutical formulations of the present invention can also include 10 veterinary compositions, e.g., pharmaceutical preparations of the *hedgehog* antagonists suitable for veterinary uses, e.g., for the treatment of livestock or domestic animals, e.g., dogs.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including 15 hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a *hedgehog* antagonist at a particular target site.

The preparations of the present invention may be given orally, parenterally, 20 topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, controlled release patch, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral and topical administrations are preferred.

The phrases "parenteral administration" and "administered parenterally" as 25 used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and 30 infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to 5 metabolism and other like processes, for example, subcutaneous administration.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

10        Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms such as described below or by other conventional methods known to those of skill in the art.

15        Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

20        The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular hedgehog antagonist employed, the age, sex, weight, condition, general 25 health and prior medical history of the patient being treated, and like factors well known in the medical arts.

30        A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels

lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the invention will be that amount of the compound that is the lowest dose effective to produce a therapeutic 5 effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

If desired, the effective daily dose of the active compound may be 10 administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure.

The patient receiving this treatment is any animal in need, including 15 primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

The compound of the invention can be administered as such or in admixtures with pharmaceutically acceptable and/or sterile carriers and can also be administered in conjunction with other antimicrobial agents such as penicillins, cephalosporins, 20 aminoglycosides and glycopeptides. Conjunctive therapy, thus includes sequential, simultaneous and separate administration of the active compound in a way that the therapeutical effects of the first administered have not entirely disappeared when the subsequent agent is administered.

25 V. Pharmaceutical Compositions

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition). The *hedgehog* antagonists according to the invention may be formulated for administration in any convenient way for use in human or

veterinary medicine. In certain embodiments, the compound included in the pharmaceutical preparation may be active itself, or may be a prodrug, e.g., capable of being converted to an active compound in a physiological setting.

Thus, another aspect of the present invention provides pharmaceutically acceptable compositions comprising a therapeutically effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam. However, in certain embodiments the subject compounds may be simply dissolved or suspended in sterile water. In certain embodiments, the pharmaceutical preparation is non-pyrogenic, i.e., does not elevate the body temperature of a patient.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect by overcoming a *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function in at least a sub-population of cells in an animal and thereby blocking the biological consequences of that pathway in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of

human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or 5 solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically 10 acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower 15 oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; 20 (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the present *hedgehog* antagonists may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically acceptable salts with pharmaceutically 25 acceptable acids. The term "pharmaceutically acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or 30 inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate,

oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19)

5 The pharmaceutically acceptable salts of the subject compounds include the conventional nontoxic salts or quaternary ammonium salts of the compounds, e.g., from non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloride, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from  
10 organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

15 In other cases, the compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable bases. The term "pharmaceutically acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the  
20 compounds, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically acceptable metal cation, with ammonia, or with a pharmaceutically acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and  
25 aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (See, for example, Berge et al., *supra*)

30 Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents,

sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, 5 sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

10 Formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to 15 produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per 20 cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are 25 prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the 30 form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension

in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an 5 active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or 10 dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain 15 silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and 20 mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

25 A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets 30 may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also 5 be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid 10 compositions that can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be 15 used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, 20 solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, 25 groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

5 It is known that sterols, such as cholesterol, will form complexes with cyclodextrins.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable 10 nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active *hedgehog* antagonist.

15 Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

20 Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required.

25 The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

30 Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the *hedgehog* antagonists in the proper medium. Absorption enhancers can also be used to increase the flux of the *hedgehog* 5 antagonists across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

10 Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior 15 to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

20 Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

25 These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium 30 chloride, and the like into the compositions. In addition, prolonged absorption of the

injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may 5 be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

10       Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot 15 injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 20 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The addition of the active compound of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compound in an effective amount and incorporating the premix into the 25 complete ration.

Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed. The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied Animal Nutrition", W.H. Freedman and CO., San

Francisco, U.S.A., 1969 or "Livestock Feeds and Feeding" O and B books, Corvallis, Ore., U.S.A., 1977).

VI. Synthetic Schemes and Identification of Active Antagonists

5 For example, many subject compounds, and congeners thereof, can be prepared readily by employing the cross-coupling technologies of Suzuki, Stille, and the like. These coupling reactions are carried out under relatively mild conditions and tolerate a wide range of "spectator" functionality.

10 *a. Combinatorial Libraries*

The compounds of the present invention, particularly libraries of variants having various representative classes of substituents, are amenable to combinatorial chemistry and other parallel synthesis schemes (see, for example, PCT WO 94/08051). The result is that large libraries of related compounds, e.g. a variegated 15 library of compounds represented above, can be screened rapidly in high throughput assays in order to identify potential *hedgehog* antagonist lead compounds, as well as to refine the specificity, toxicity, and/or cytotoxic-kinetic profile of a lead compound. For instance, *ptc*, *hedgehog*, or *smoothened* bioactivity assays, such as may be developed using cells with either a *ptc* loss-of-function, *hedgehog* gain-of- 20 function, or *smoothened* gain-of-function, can be used to screen a library of the subject compounds for those having agonist activity toward *ptc* or antagonist activity towards *hedgehog* or *smoothened*.

Simply for illustration, a combinatorial library for the purposes of the present invention is a mixture of chemically related compounds that may be screened 25 together for a desired property. The preparation of many related compounds in a single reaction greatly reduces and simplifies the number of screening processes that need to be carried out. Screening for the appropriate physical properties can be done by conventional methods.

Diversity in the library can be created at a variety of different levels. For instance, the substrate aryl groups used in the combinatorial reactions can be diverse in terms of the core aryl moiety, e.g., a variegation in terms of the ring structure, and/or can be varied with respect to the other substituents.

5        A variety of techniques are available in the art for generating combinatorial libraries of small organic molecules such as the subject *hedgehog* antagonists. See, for example, Blondelle et al. (1995) *Trends Anal. Chem.* 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; the ArQuile U.S. Patents 5,736,412 and 5,712,171;

10      10 Chen et al. (1994) *JACS* 116:2661; Kerr et al. (1993) *JACS* 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242). Accordingly, a variety of libraries on the order of about 100 to 1,000,000 or more diversomers of the subject *hedgehog* antagonists can be synthesized and screened for particular activity or property.

15      15 In an exemplary embodiment, a library of candidate *hedgehog* antagonists diversomers can be synthesized utilizing a scheme adapted to the techniques described in the Still et al. PCT publication WO 94/08051, e.g., being linked to a polymer bead by a hydrolyzable or photolabile group, optionally located at one of the positions of the candidate antagonists or a substituent of a synthetic intermediate.

20      20 According to the Still et al. technique, the library is synthesized on a set of beads, each bead including a set of tags identifying the particular diversomer on that bead. The bead library can then be “plated” with *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function cells for which an *hedgehog* antagonist is sought. The diversomers can be released from the bead, e.g., by hydrolysis.

25

#### Biological Assays

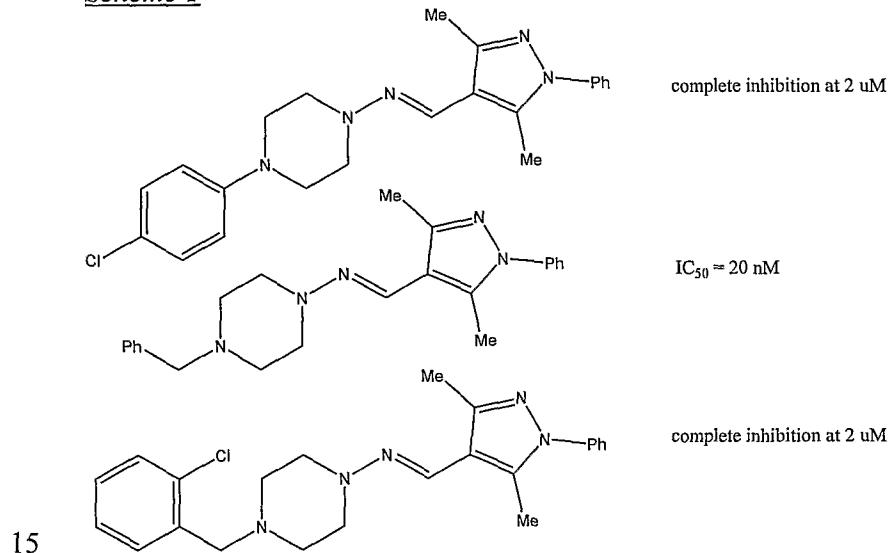
##### *Lead Compound Discovery/ High-throughput Screening Assay*

Compounds (10,000) were acquired from Chembridge (San Diego) as DMSO solutions in 96-well format. Shh-N (N-terminal fragment of Shh without 30 cholesterol modification)-conditioned medium was obtained from an HEK 293 cell line stably transfected with Shh-N expression and neomycin resistance constructs.

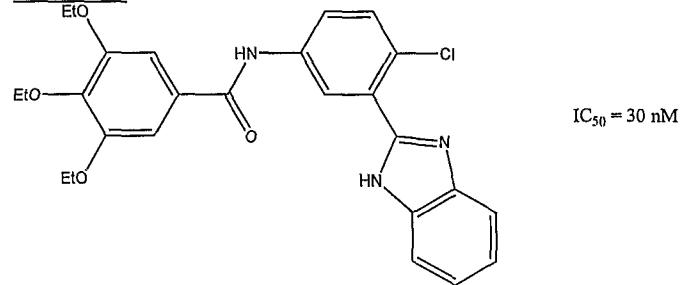
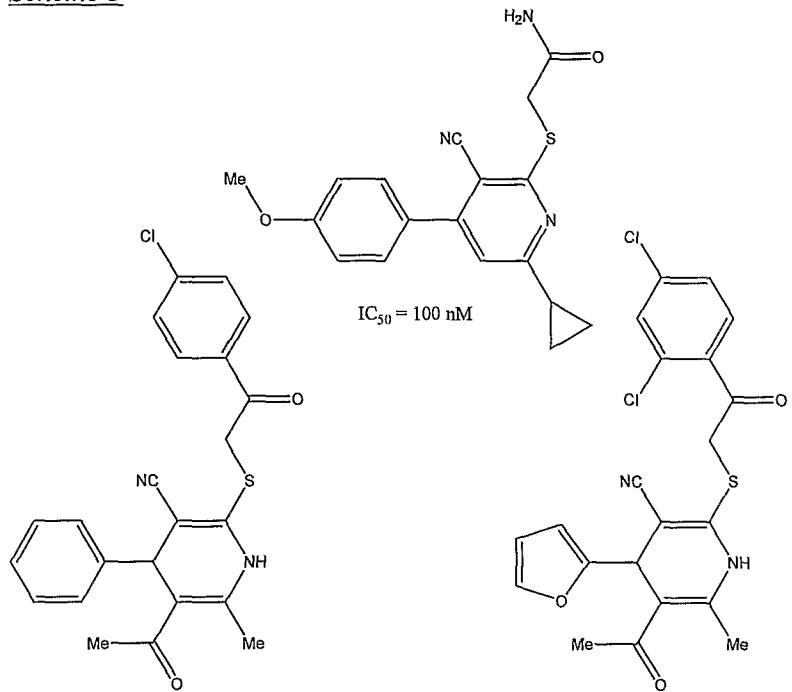
The Shh-N-producing HEK 293 cells were grown to 80% confluence in DMEM containing 10% (vol/vol) FBS and 400 µg/ml G418. The medium then was replaced with DMEM containing 2% (vol/vol) FBS, and after 1 day of growth, the medium was collected and filtered through a 0.22-µm membrane. Control medium was obtained from HEK 293 cells. Shh-LIGHT2 cells were then cultured to confluence in 96-well plates and treated with the small molecules (0.714 µg/ml;  $\approx$ 2 µM compound in each well) in the presence of either Shh-N-conditioned medium or HEK 293 control medium (1:25 dilution into DMEM containing 0.5% bovine calf serum). After incubating the treated cells for 30 h at 37 °C, cellular firefly and *Renilla* luciferase activities were measured (Taipale et al., *Nature* 406:1005-1009 (2000)).

Compounds identified in the above assay are depicted below in Schemes 1-6.

Scheme 1

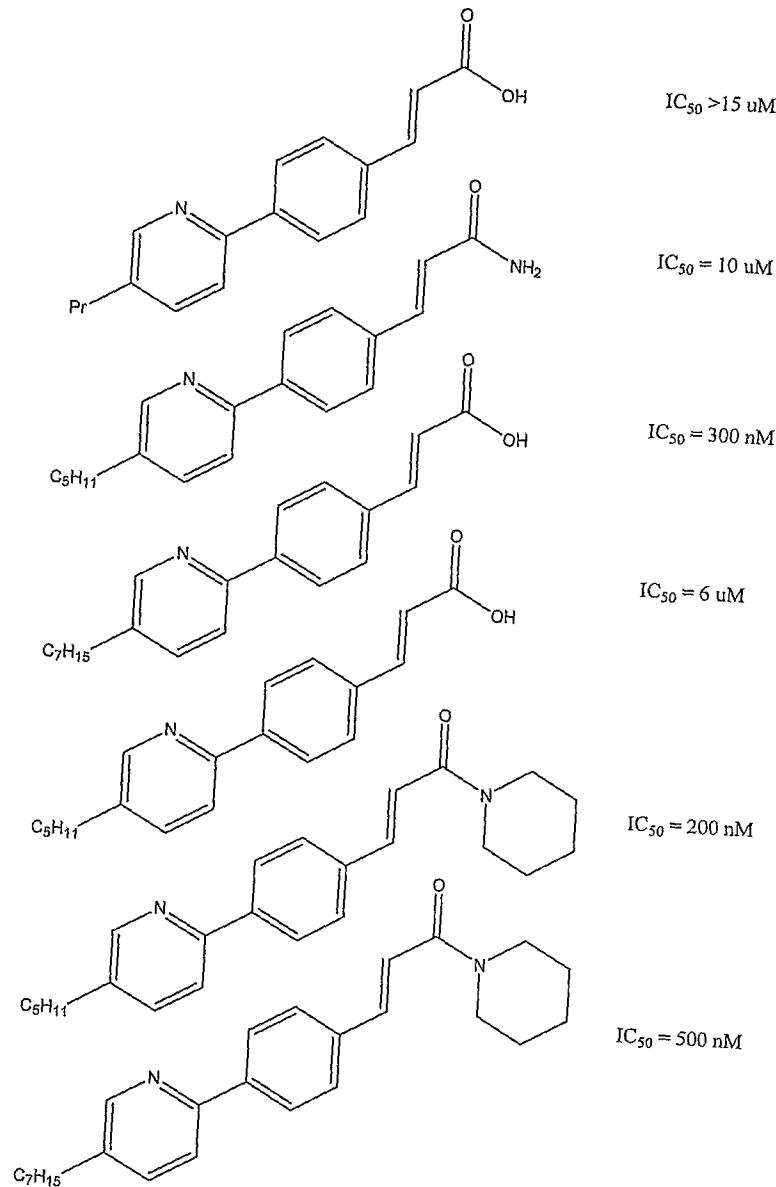


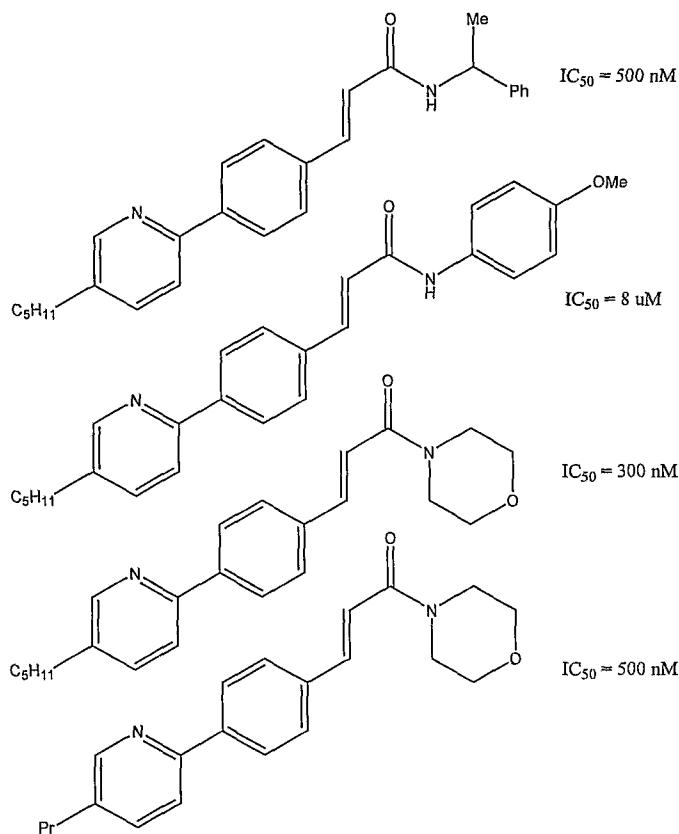
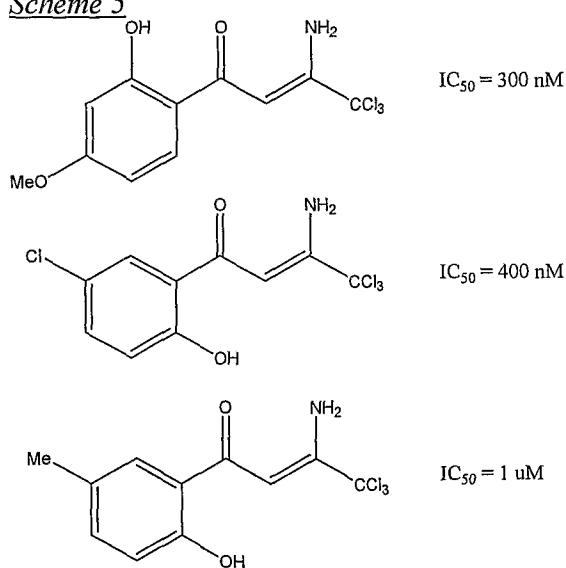
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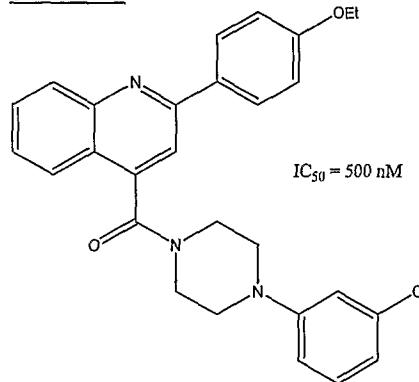
Scheme 2 $IC_{50} = 30 \text{ nM}$ Scheme 3

5

 $IC_{50} > 15 \text{ uM}$  $IC_{50} = 1 \text{ uM}$

Scheme 4

Scheme 5

Scheme 6

The compounds of Schemes 5 and 6 inhibit the hedgehog pathway

5 downstream of *smoothened*, and thus may effectively inhibit the hedgehog pathway in cells that have a *smoothened* gain-of-function mutation that attenuates the activity of compounds that act directly on *smoothened* or renders them ineffective.

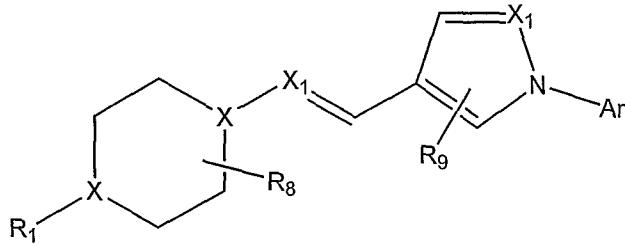
All of the publications and references cited above, as well as U.S. Provisional Applications Nos. 60/374,371, filed 4/22/02, 60/405,689, filed 8/23/02, 10 and 60/415,822, filed 10/03/02, are hereby incorporated by reference herein.

### *Equivalents*

Those skilled in the art will recognize, or be able to ascertain using no more 15 than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

**Claims:**

1. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula (I):



5

**Formula I**

wherein, as valence and stability permit,

Ar represents a substituted or unsubstituted aryl or heteroaryl ring;

X represents, independently for each occurrence, N or CR';

X<sub>1</sub> represents, independently for each occurrence, N or CR'';

10 R' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate;

15 R'' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate;

20 R<sub>1</sub> represents a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heterocyclyl alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl;

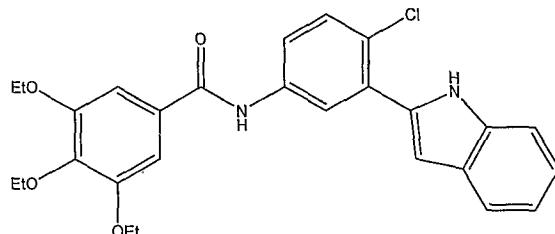
R<sub>8</sub> represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl,

thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate; and

$R_9$  represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, or a pharmaceutically acceptable salt thereof.

10 2. A composition of claim 1, wherein Ar represents a substituted or  
unsubstituted phenyl ring, X and X<sub>1</sub> represent N for all occurrences, R<sub>1</sub> represents  
alkyl, heteroaralkyl, or aralkyl, R<sub>8</sub> represents 0 substituents, and R<sub>9</sub> represents two  
methyl groups separately located on the two unsubstituted carbon atoms of the ring  
to which it is attached.

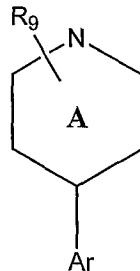
15 3. A pharmaceutical composition comprising a compound having the structure



, or a pharmaceutically acceptable salt

thereof.

4. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula II:



20

Formula II

wherein, as valence and stability permit,

A represents a pyridyl or dihydropyridyl ring;

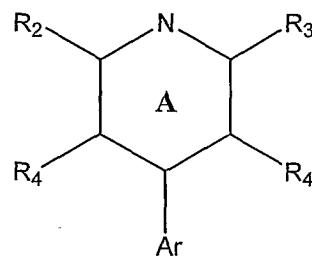
Ar represents a substituted or unsubstituted aryl or heteroaryl ring; and

5 R<sub>9</sub> represents from 0-4 substituents on the ring to which it is attached, independently selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and

10 carbonate,

or a pharmaceutically acceptable salt thereof.

5. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula III:

Formula III

15 wherein, independently for each occurrence,

A represents a pyridyl or dihydropyridyl ring;

Ar represents a substituted or unsubstituted aryl or heteroaryl ring; and

R<sub>2</sub> represents a thioether, preferably substituted with an amido, ester, carboxyl, or

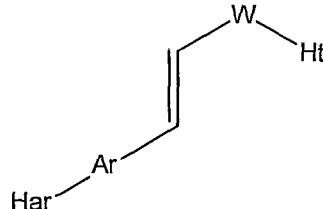
20 acyl;

R<sub>3</sub> represents H, alkyl, alkenyl, alkynyl, or cycloalkyl; and

R<sub>4</sub> represents a substituent selected from H, halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride,

silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate, or a pharmaceutically acceptable salt thereof.

6. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula IV:

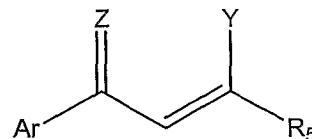


Formula IV

wherein, as valence and stability permit,  
 Ar represents a substituted or unsubstituted aryl or heteroaryl ring;  
 10 W represents C=O, C=S, or SO<sub>2</sub>;  
 Har represents a substituted or unsubstituted heteroaryl ring;  
 Ht represents OR or NR'''<sub>2</sub>;  
 R represents H or lower alkyl; and  
 R''' represents H, lower alkyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heterocyclyl  
 15 alkyl, aryl, aralkyl, heteroaryl, or heteroaralkyl,  
 or a pharmaceutically acceptable salt thereof.

7. A composition of claim 6, wherein Ar represents a substituted or unsubstituted phenyl ring, and Har represents a substituted or unsubstituted 2-pyridyl ring.

20 8. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula V:



Formula V

wherein, as valence and stability permit,

Ar represents a substituted or unsubstituted aryl or heteroaryl ring, preferably a substituted or unsubstituted phenyl ring, such as an ortho-hydroxyphenyl group optionally substituted with one or more halogen, hydroxy, alkoxy, amino, alkylamino, alkyl, thiol, alkylthio, ester, amido, carboxyl, nitro, or cyano substituents;

Y represents OH or NR<sub>2</sub>;

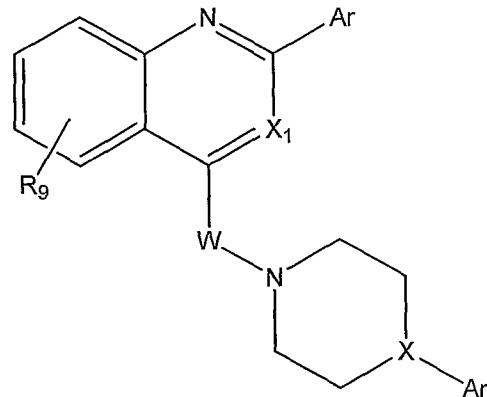
Z represents O or S;

R represents H or lower alkyl; and

10 R<sub>5</sub> represents substituted or unsubstituted lower alkyl, or a pharmaceutically acceptable salt thereof.

9. A composition of claim 8, wherein R<sub>5</sub> represents a trihalomethyl group, Y represents NH<sub>2</sub>, and Ar represents an optionally further substituted 2-hydroxyphenyl substituent.

15 10. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a compound having a structure of Formula VI:



Formula VI

wherein, as valence and stability permit,

20 Ar, independently for each occurrence, represents a substituted or unsubstituted aryl or heteroaryl ring;

W represents C=O, C=S, or SO<sub>2</sub>;

X represents, independently for each occurrence, N or CR';  
X<sub>1</sub> represents, independently for each occurrence, N or CR'';  
R represents H or lower alkyl; and  
R' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, alkynyl,

5       aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate;

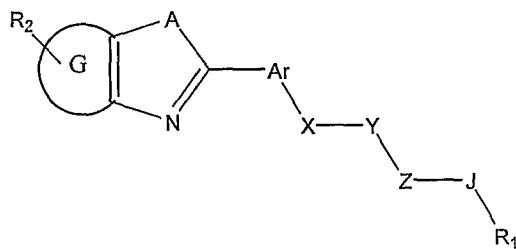
R'' represents, independently for each occurrence, H, halogen, alkyl, alkenyl, 10       alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, alkylamino, nitro, cyano, thiol, amino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, or carbonate; and

R<sub>9</sub> represents from 0-4 substituents on the ring to which it is attached, independently 15       selected from halogen, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, silyloxy, amino, nitro, cyano, thiol, amino, alkylamino, imino, amido, phosphoryl, phosphonate, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, selenoether, acyl, aldehyde, ester, carbamate, and carbonate,

20       or a pharmaceutically acceptable salt thereof.

11.       A composition of claim 10, wherein Ar represents a substituted or unsubstituted phenyl ring, X represents N, X<sub>1</sub> represents CR, and R<sub>9</sub> is absent or represents 1-4 substituents selected from halogen, alkyl, cyano, and nitro.

12.       A pharmaceutical composition comprising a pharmaceutically 25       acceptable excipient and a compound having a structure of Formula VII:



wherein, as valence and stability permit,

10 X and Z, independently, represent -N(R<sub>7</sub>)-, -O-, -S-, -(R<sub>7</sub>)N-N(R<sub>7</sub>)-, -ON(R<sub>7</sub>)-, or a direct bond;

5 Y represents -C(=O)-, -C(=S)-, -C(=NR<sub>7</sub>)-, SO<sub>2</sub>, or SO;

A represents O, S, or NR<sub>7</sub>;

G represents a cycloalkyl, heterocyclyl, aryl, or heteroaryl ring fused to the ring to which it is attached;

15 Ar represents a substituted or unsubstituted aryl or heteroaryl ring;

R<sub>1</sub> represents H or substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, heteroaryl, heterocyclyl, or cycloalkyl, including polycyclic groups;

R<sub>2</sub> represents from 0-4 substituents on the ring to which it is attached, such as halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl group,

20 thiocarbonyl, ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, J-R<sub>8</sub>, J-OH, J-lower alkyl, J-lower alkenyl, J-R<sub>8</sub>, J-SH, J-NH<sub>2</sub>, protected forms of the above, or any two R<sub>2</sub>, when occurring more than once in a cyclic or polycyclic structure, can be taken together form a 4- to 8-membered cycloalkyl, aryl, or heteroaryl;

25 R<sub>7</sub>, independently for each occurrence, represents H or substituted or unsubstituted lower alkyl, J-cycloalkyl, J-heterocyclyl, J-aryl, or J-heteroaryl;

R<sub>8</sub>, independently for each occurrence, represents H or substituted or unsubstituted lower alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl; and

J represents, independently for each occurrence, a chain having from 0-8 units selected from CK<sub>2</sub>, NK, O, and S, wherein K represents, independently for each occurrence, H or lower alkyl.

13. The composition of any preceding claim, wherein the compound inhibits *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function mediated signal transduction with an IC<sub>50</sub> of 1  $\mu$ M or less.
14. A method of inhibiting the *hedgehog* pathway in a cell, comprising  
5 contacting the cell with a composition of any preceding claim.
15. The method of claim 14, wherein the cell has a phenotype of *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function.
16. The method of claim 14, wherein the cell is contacted with the *hedgehog* antagonist *in vivo*.
- 10 17. The method of claim 14, wherein the cell is contacted with the *hedgehog* antagonist *in vitro*.
18. The method of claim 14, wherein the compound is administered to an animal as part of a therapeutic or cosmetic application.
19. The method of claim 18, wherein the therapeutic or cosmetic application is  
15 selected from regulation of neural tissues, bone and cartilage formation and repair, regulation of spermatogenesis, regulation of smooth muscle, regulation of lung, liver and other organs arising from the primitive gut, regulation of hematopoietic function, and regulation of skin and hair growth.
- 20 20 A method for inhibiting unwanted proliferation of a cell, comprising  
20 contacting the cell with a composition of any of claims 1-13.
21. The method of claim 20, wherein the cell is a cancer cell.
22. The method of claim 21, wherein the cell is a cancer cell from a cancer selected from medulloblastoma, rhabdomyosarcoma, endodermally or gut-derived cancers (including small cell lung cancer, esophageal cancer, gastric (stomach)

cancer, biliary cancer, and pancreatic cancer), urogenital cancers (including prostate cancer and bladder cancer), ovarian cancer, and uterine cancer.

23. A method for treating or preventing a carcinoma, comprising administering a composition of any of claims 1-13 to a patient in an amount sufficient to inhibit  
5 progression of the carcinoma.

24. The method of claim 23, wherein the carcinoma is a basal cell carcinoma.

25. A method of inhibiting the *hedgehog* pathway in a cell, comprising contacting the cell with a compound that inhibits activation of the *hedgehog* pathway downstream of *smoothened*.